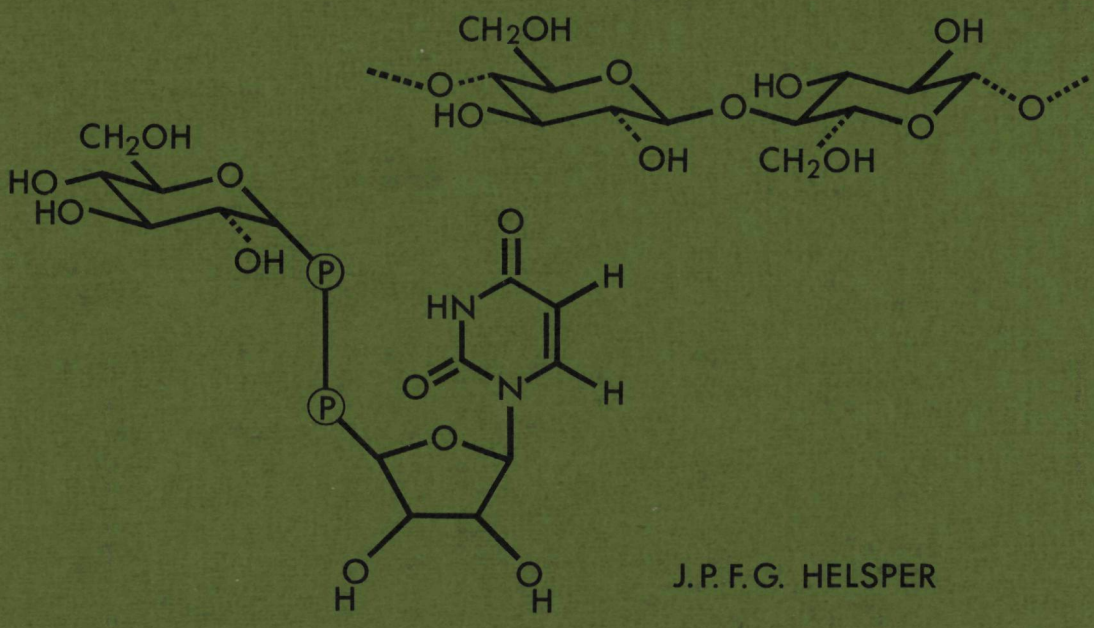
β-Glucan Synthesis by a Membrane Fraction from Germinating *PETUNIA* Pollen



J.P.F.G. HELSPER

Errata

Pagina	Regel	
VI	17	Veerlamp = Veerkamp
VIII	9	22 = 23
2	10	lattice for lat- = lat-
23	2	3.2. = 3.1
24	1	cyrochrome = cytochrome
50	12	1 = 15, 15 = 1
54	2	2.7 = 3.7
	4	8.1 = 11.1
	5, 8	4 = 5.5

β-GLUCAN SYNTHESIS BY A MEMBRANE FRACTION FROM GERMINATING *PETUNIA* POLLEN

PROMOTORES

Dr. M. M. A. Sassen Dr. J. H. Veerkamp

β-GLUCAN SYNTHESIS BY A MEMBRANE FRACTION FROM GERMINATING *PETUNIA* POLLEN

PROEFSCHRIFT

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door

JOHANNES PETRUS FRANCISCUS GERARDUS HELSPER

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Ter nagedachtenis aan mijn vader

Aan mijn moeder

Aan Annelies en Ellen

Aan het begin van dit proefschrift wil ik mijn dank betuigen aan iedereen die aan de totstandkoming hiervan heeft meegewerkt.

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The investigations described in this thesis were carried out at the Department of Botany, University of Nijmegen, Nijmegen, The Netherlands, under the direction of Dr. M.M.A. Sassen and Dr. J.H. Veerlamp (Department of Biochemistry)

ATP	adenosine triphosphate
Ci	Curie (1 Curie = 2.2 x 10^{12} dpm)
cpm	counts per minute
DEAE	diethylaminoethyl
dpm	disintegrations per minute
EDTA	ethylenediamine tetraacetate
GDP	guanosine diphosphate
HIO_4-PTA/CrO_3	1% periodic acid-1% phosphotungstic acid/ 10% chromic acid
H_2O_2/CH_3COOH	30% hydrogen peroxide/glacial acetic acid (1:1 v/v)
IDP	inosine diphosphate
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
rpm	revolutions per minute
TRIS	tris(hydroxymethyl)aminomethane
UDP	uridine diphosphate

General introduction

1.1. Cellulose

Each year photoautotrophic plants produce 10^{11} tons of cellulose (Colvin, 1972). It is the most synthesized product on earth, followed by chitin with 10^{9} tons per year. Chitin is chemically related to cellulose, but is synthesized mainly in photoheterotrophic organisms such as fungi and insects. With pectins, hemicelluloses and hydroxyproline-rich glycoproteins cellulose forms the cell wall of plants. In these cell walls cellulose forms a fibrillar network, which is embedded in an amorphous matrix composed of the

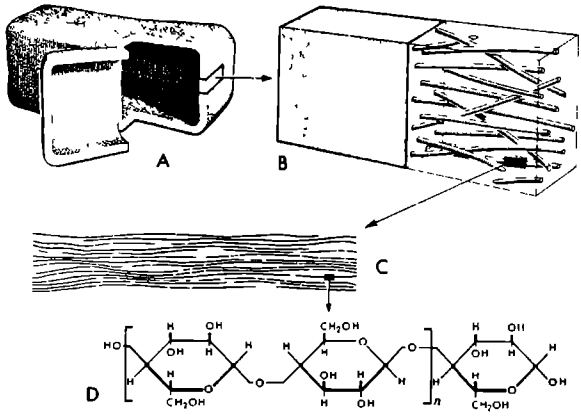


Fig. 1.1. Schematic representation of the molecular organisation of cellulose in cell walls of plants. A. Partly opened cell wall. B. Amorphous matrix with cellulose microfibrils. C. One cellulose microfibril, composed of β-1,4-glucan chains. D. One β-1,4-glucan chain. Reprinted from Sassen (1971)

other cell wall constituents (Fig. 1.1). The function of cellulose is to stabilize and fortify the amorphous matrix like steel rods in reinforced concrete. Man uses cellulose for a wide variety of applications. It is the main constituent of paper, cotton and wood. It is made into rayon, cellophane and nitrocellulose. In

nature upon prolonged storage under high temperature and pressure cellulose is converted into coal and other fossil fuel

Besides to more technological applications, research has been carried out with regard to structure, synthesis and degradation (Coughlan and Folan, 1979) of cellulose. Electron microscopical studies established that cellulose occurs as microfibrillar structures (Frey-Wyssling et al., 1948, Preston et al , 1948), partly crystallized aggregates of β-1,4-glucan chains varying in width from 3.5 (elementary fibrils) to 30 nm (Frey-Wyssling, 1976, p. 16). X-ray diffraction revealed a monoclinic lattice for lattice for cellulose with a fiber period, parallel to the β-glucan chains, of 1.03 nm (b-axis) and periods varying from 0.8 to 1.6 nm for both the a- and c-axis (Frey-Wyssling, 1976, p. 93). The degree of polymerization of native cellulose varies from 2,000 to 16,000 (Frey-Wyssling, 1976, p 91). Parallel or antiparallel orientation of the β-glucan chains within the fibrils is still an uncertain aspect of cellulose structure.

1.2. Orientation of cellulose microfibrils

Two theories exist about the mechanisms that cause the orientation of microfibrils during cell wall growth, the 'multinet growth hypothesis' and the ordered fibril hypothesis'. The multinet growth hypothesis postulates a passive reorientation in the longitudinal direction of originally more transversely deposited microfibrils (Roelofsen and Houwink, 1953). According to the ordered fibril hypothesis of Roland et al. (1975) the orientation of fibrillar polysaccharides is determined at the time of deposition. Linearly arranged particles in the plasma membrane and/or microtubules underlying this membrane are suggested as the structures responsible for the direction in which the microfibrils are deposited (Brown and Montezinos, 1976, Mueller et al., 1976; Palevitz and Hepler, 1976; Willison and Brown, 1977, 1978, Robenek and Peveling, 1977). Some authors postulate that these particles are also involved in the synthesis of microfibrils (Brown and Montezinos, 1976, Willison and Brown, 1977, 1978).

1.3. *Sites of cellulose synthesis*

Synthesis of β-1,4-glucan chains and the subsequent crystallization to microfibrils are processes which are supposed to occur outside the cell on the plasma membrane (Colvin, 1972; Albersheim, 1978). However, in *Pleurochrysis scherffelii* cellulose is observed within Golgi vesicles (Brown et al., 1969, 1970, 1973; Herth et al., 1972). Apparently the synthesis and crystallization also take place at this site. Ray et al. (1969) observed β-glucan synthetase activity in a particulate enzyme system, identified as Golgi membranes by its IDPase activity. Engels (1973) isolated a membrane fraction from germinating *Petunia* pollen, which was characterized on morphological basis as Golgi vesicles. After chloroform and alkali extraction of this fraction he obtained from the insoluble material an X-ray diffraction pattern, typical for cellulose (Engels, 1974b).

1.4. *In vitro synthesis of cellulose*

The synthesis of the building blocks for cellulose microfibrils, the β-1,4-glucan chains, was studied mainly *in vitro*. The first study of β-glucan synthesis has been reported by Glaser (1957) with a particulate enzyme system from *Acetobacter xylinum*, a Gram-negative bacterium. Elbein et al. (1964) were the first who reported β-glucan synthesis *in vitro* in higher plants with an enzyme system from *Phaseolus aureus*.

Lipid intermediates are a common phenomenon in the synthesis of extracellular polysaccharides (Waechter and Lennarz, 1976; Hemming, 1977, 1978). The lipids may serve as carriers for activated mono- and oligosaccharides through the membrane. They have a polyprenol phosphate nature with 10-20 isoprenyl residues, of which the α-residue should be saturated for optimal activity (Mańkowski et al., 1975, 1977). Polyprenol phosphate glucosylation is catalysed by particulate enzyme preparations from *Acetobacter xylinum* (Kjosbakken and Colvin, 1973; Garcia et al., 1974) and by enzymes of the Golgi system of *Pisum sativum* (Lezica et al., 1976),

Phaseolus aureus (Bowles et al., 1977), *Allium cepa* and *Calendula officinalis* (Lercher and Wojciechowski, 1976). Up to now only Hopp et al. (1978) obtained more conclusive evidence for the involvement of such lipid intermediates in β-glucan synthesis *in vitro* in a particulate enzyme system from *Prototheca zopfii*.

Forge (1977) is the only one claiming the *in vitro* synthesis of cellulose microfibrils with a particulate preparation from *Acetobacter xylinum*. However, Schnepf and Herth (1978) doubt the validity of this observation.

1.3 Aims of this study

This thesis is an extension of the work of Engels (1973, 1974a, 1974b). It deals with investigations about the presence and mechanisms of β-glucan synthesis in a membrane fraction from germinating *Petunia* pollen, similar to that used by Engels.

The materials and methods, applied in our studies, are described in chapter 2.

Chapter 3 deals with the characterization of the membrane fraction by means of marker enzyme activities and the HIO_4-PTA/CrO_3 staining technique according to Roland et al. (1972). The selectivity of this staining technique for plasma membranes of plant cells (Roland et al., 1972, Roland, 1978, Nagahashi et al., 1978) is tested on germinating *Petunia* pollen *in situ*.

Chapter 4 concerns the biochemical characterization of the β-glucan synthetase activity in the membrane fraction. The newly-synthesized β-glucans have been investigated for the presence of β-1,3- and β-1,4-glucosidic linkages.

The investigation of the possible role of lipid intermediates in β-glucan synthetase activity of this membrane fraction is described in chapter 5.

In chapter 6 an electron microscopic autoradiographic investigation is described of the *in vitro* synthesized β-glucans. With this technique we hoped to find out wether these β-glucans crystallize to microfibrils.

Chapter 2

Materials and Methods

2.1. Preparations

Germinating *Petunia* pollen were obtained by growing pollen from *Petunia hybrida*, strain W166K, for 90 min at 25°C as described by Schrauwen and Linskens (1967).

Crude pollen tube preparations were obtained by homogenizing germinating *Petunia* pollen in 0.3 M sucrose, buffered with 0.1 M phosphate (pH 7.2) containing 4 mM Na_2EDTA. The pellet was sedimented for 1 h at 46,000 rpm in a SW 50^{I} Beckman rotor. The pellet was washed twice with the same buffer. After each wash the homogenate was centrifuged as described above. The final pellet is the "crude pollen tube preparation".

A rough membrane suspension was obtained by centrifugation of the homogenate of germinating *Petunia* pollen for 5 min at 2,500 rpm to remove nuclei and cell walls. This suspension was layered on a discontinuous sucrose gradient composed of four cushions with the following concentrations: 0.5 M, 1.0 M, 1.5 M and 2.0 M sucrose in the above mentioned buffer. This gradient was centrifuged for 70 min at 27,000 rpm in a SW 27 Beckman rotor. Membrane fractions were collected from the 0.3-0.5 M, 0.5-1.0 M, 1.0-1.5 M, and the 1.5-2.0 M sucrose interspaces. The membrane fraction, collected from the 0.5-1.0 M sucrose interspace is further referred to as "the membrane fraction".

To obtain the membrane material from the 0.7-0.9 M sucrose interspace, the membrane fraction from the 0.5-1.0 M sucrose interspace was diluted with the above mentioned buffer and centrifuged for 70 min at 27,000 rpm in a SW 27 Beckman rotor on a second sucrose gradient composed of four cushions of 0.5 M, 0.7 M, 0.9 M and 1.1 M sucrose in the above mentioned buffer. All mem-

brane suspensions were sedimented by centrifugation for 1 h in a SW 50.1 Beckman rotor at 46,000 rpm.

2.2. Incubation and extraction procedures

Unless otherwise mentioned, all incubations were carried out at 25°C in 0.1 M TRIS-HCl (pH 8.0), 20 mM $MgCl_2$, 10 mM cellobiose, 4 mM Na_2EDTA and 1 mM dithiothreitol at a final concentration of 1 mg protein/ml.

2.2.1. Isolation of alkali-insoluble material for β-glucan synthetase assays

For β-glucan synthetase assays membrane material equivalent to 0.1 mg protein was incubated for 90 min with 5.8 nmoles UDP-[^{14}C]glucose (30 nCi) or 5.8 nmoles GDP-[^{14}C]glucose (300 nCi). For electron microscopic autoradiography the membrane fraction equivalent to 10 mg protein was incubated for 90 min with 0.03 nmoles UDP-[^{3}H]glucose (100 μCi). The reactions were stopped by heating for 5 min at 95°C. In β-glucan synthetase assays 0.9 ml water (95°C) was added together with 10 mg powdered cellulose, which acts as a carrier.

The precipitate was extracted 3 times with water (95°C), once with chloroform-methanol, once with methanol and twice with 1 N NaOH (95°C). The residual pellet was rinsed once with water to give the "alkali-insoluble material". The radioactivity in the alkali-insoluble material is taken as a measure for β-glucan synthetase activity. The combined chloroform-methanol and methanol extracts are referred to as "lipid-soluble material" and the NaOH extracts combined with the water rinse as the "alkali-insouble material".

For electron microscopic autoradiography the alkali-insoluble material was suspended in 0.85 ml water. A 0.2 ml-sample of this suspension was treated for 30 s in a MSE PG-100 ultrasonic disintegrator. A second sample of 0.2 ml was treated with 1.8 ml 30%

hydrogen peroxide/glacial acetic acid (1:1 v/v) for 2 h at 100°C (H_2O_2/CH_3COOH treatment). The insoluble material was washed three times with 1 ml water. On a third sample ultrasonic disntegration, immediately followed by H_2O_2/CH_3COOH treatment, was carried out. After the H_2O_2/CH_3COOH treatments and washings the pellets were suspended in 0.2 ml water.

2.2.2. Investigations on the role of lipid intermediates in β-glucan synthesis

In these investigations the membrane fraction equivalent to 0.2 mg protein was incubated in various ways and after the incubations various extraction procedures were applied.

In characterization studies membranes were incubated for 30 min with 5 μCi UDP-[^{3}H]glucose (3.7 Ci/mmole). Figure 2.1 shows a schematic representation of the extraction procedure. The reaction was stopped by addition of 0.8 ml methanol, containing 10 mg powdered cellulose. After shaking and extraction for 5 min at 56°C the mixture was centrifuged for 10 min at 2,500 rpm. The pellet was extracted twice with 0.5 ml 80% methanol at 56°C for 5 min and once with 0.6 ml $CHCl_3$-CH_3OH (1:2 v/v). The final pellet is referred to as pellet 1. To the combined supernatants 1.4 ml distilled water and 1.8 ml $CHCl_3$ were added and the solutions were thouroughly mixed. The organic phase plus interphase and the methanol-water phase were separated by centrifugation for 5 min at 2,500 rpm. The organic phase plus interphase were washed twice with 1 ml $CHCl_3$-CH_3OH-H_2O (3:47:48 by vol), evaporated to dryness under N_2 and redissolved in 0.2 ml $CHCl_3/CH_3OH$ (2:1 v/v). This fraction is referred to as the crude lipid preparation. The two washes with $CHCl_3$-CH_3OH-H_2O (3:47:48 by vol) were combined with the methanol-water phase and referred to as the methanol-water phase.

In incorporation studies with isolated lipids, crude lipid preparation (30,000 cpm) and polar lipids (1,200 cpm) were evaporated under N_2 and solubilized in Triton X-100 (0.04-4.0%). The Triton X-100 solubilized lipids were diluted four times with

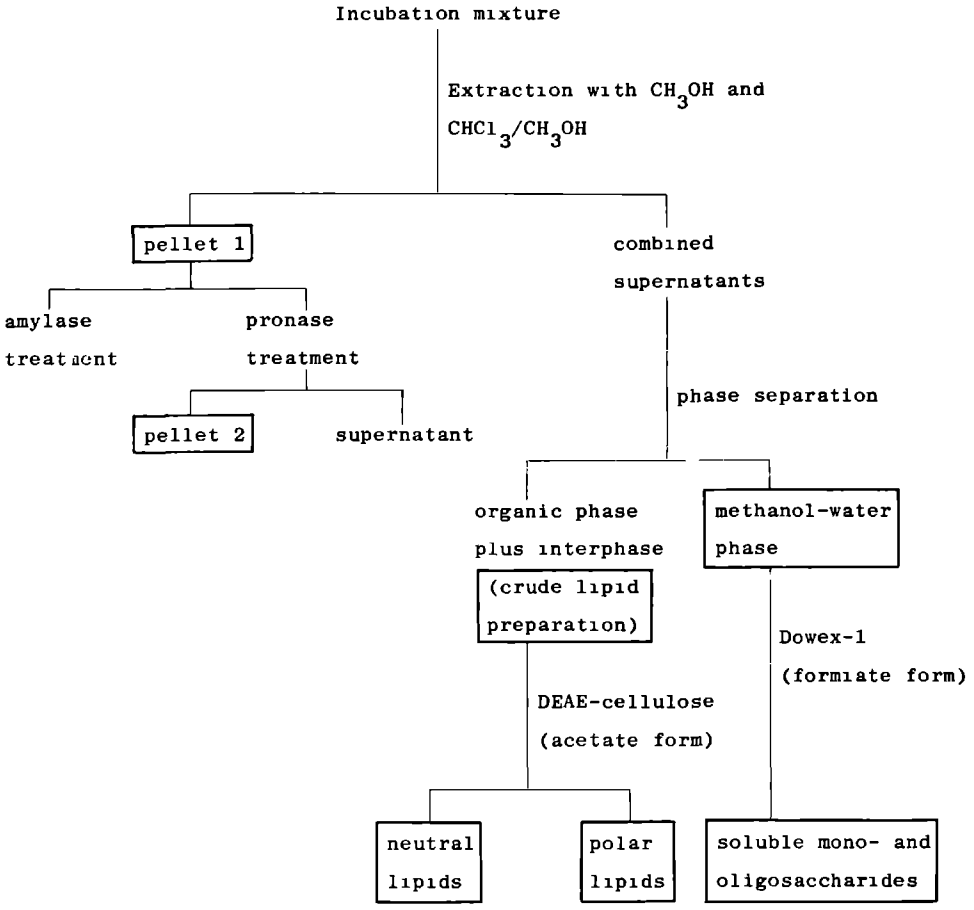


Fig. 2.1. Schematic representation of the chromatographic and extraction procedures, applied in characterization studies, kinetic experiments and double-labelling experiments. The fractions, enclosed by lines, were characterized further

the incubation buffer and the membrane suspension. After 90 min the reactions were stopped by heating the reaction mixture to 95°C for 5 min. Then 0.8 ml water was added together with 10 mg of powdered cellulose. After precipitation the pellet was extracted to alkali-insoluble material as described in 2.2.1.

In kinetic experiments the initial synthesis rates of neutral lipids, polar lipids and pellet 2 were determined at UDP-glucose concentrations, varying from 2 to 100 µM UDP-[^{14}C]glucose (225 cpm/pmole). The extraction procedure was as described for characterization studies. Pellet 2 was obtained as described in "characterization of pellet 1". Polar lipids were separated from neutral lipids as described below ("characterization of the crude lipid preparation") with the exception that the polar lipids were eluted from the DEAE cellulose column with 6 ml 0.1 M ammonium acetate/99% methanol immediately after the column was eluted with $CHCl_3/CH_3OH$ (2:1 v/v) and with CH_3OH. The eluates were collected in scintillation vials and evaporated under streaming air at 56°C.

In double-labelling experiments 1 µCi UDP-[^{14}C]glucose (312 mCi/mmole) was added after 30 min incubation with 5 µCi UDP-[^{3}H]glucose (3.7 Ci/mmole) The reactions were stopped at 1,2 or 5 min after application of UDP-[^{14}C]glucose by addition of 0.3 ml CH_3OH , containing 10 mg of powdered cellulose. The extraction procedure was as described for characterization studies. Pellet 2 was obtained as described in "characterization of pellet 1". Polar lipids were separated by thin-layer chromatography in solvent A ($CHCl_3$-CH_3OH-H_2O 62.25 3 by vol). After development 0.5 cm-bands were scraped off from the chromatogram and the radioactive compounds were eluted with 1.5 ml CH_3OH for 1 h. The $^{3}H/^{14}C$ ratios at Rf of 0.2 represents the $^{3}H/^{14}C$ ratio in polar lipids and that at Rf of 0.4-0.85 the $^{3}H/^{14}C$ ratio in neutral lipids.

2.3. Characterization of the crude lipid preparation

The crude lipid preparation was separated by thin-layer chromatography in solvent A or by chromatography on a DEAE cellulose

column (acetate form, 50 x 4 mm). The column was first eluted with 6 ml $CHCl_3$-CH_3OH (2:1 v/v) to obtain the neutral lipids. Subsequently the column was eluted with 6 ml CH_3OH. To obtain the polar lipids this column was then eluted with five 18 ml-fractions of 99% CH_3OH, containing 0.01 M; 0.03 M; 0.1 M; 0.2 M and 0.4 M ammonium acetate. To remove ammmonium acetate from the polar lipids, the radioactive fraction was concentrated under N_2 to 2 ml. Then 1.8 ml distilled water and 2 ml $CHCl_3$ were added. After shaking and centrifugation for 5 min at 2,500 rpm the ammonium acetate was removed together with the upper phase. The organic phase plus interphase were washed twice with $CHCl_3$-CH_3OH-H_2O (3:47:48 by vol).

2.3.1. Alkaline hydrolysis of neutral lipids

After evaporation to dryness the neutral lipids were hydrolysed in 1 ml 50% CH_3OH, containing 0.1 N NaOH, for 15 min at 37°C. After this treatment 1.0 ml 0.1 N HCl was added and after shaking this was followed by the addition of 1.5 ml CH_3OH, 0.3 ml distilled water and 2 ml $CHCl_3$. The solution was thoroughly mixed and centrifuged for 5 min at 2,500 rpm. The organic phase plus interphase, containing unhydrolysed lipids, were separated from the upper phase.

2.3.2. Mild acid hydrolysis of polar lipids

Polar lipids, free from ammonium acetate, were evaporated to dryness and hydrolysed for 15 min at 100°C in 99% propanol, containing 0.01 N HCl. Then 1.0 ml CH_3OH, 1.8 ml distilled water and 2 ml $CHCl_3$ were added. The solution was thouroughly mixed and centrifuged for 5 min at 2,500 rpm. The organic phase plus interphase were separated from the upper phase and washed twice with 1 ml $CHCl_3$-CH_3OH-H_2O (3:47:48 by vol). The combined upper phases were evaporated to dryness under reduced pressure, redissolved in 100 µl distilled water and submitted to thin-layer chromatography in solvent D (n-butanol-pyridine-water 8:2:1 by vol).

2.4. Characterization of compounds in the methanol-water phase

The methanol-water phase was investigated for the presence of radioactivity in UDP-glucose and soluble mono- and oligosaccharides.

For the determination of the radioactivity in UDP-glucose the methanol-water phase was submitted to thin-layer chromatography in solvent E (M ammonium acetate-ethanol 2·5 v/v) with soluble cellodextrins and UDP-glucose as references. After development the 0.5 cm-bands, containing UDP-glucose, were scraped off from the chromatogram and the radioactive compounds were eluted for 1 h with 1.5 ml water.

For the determination of radioactivity in soluble mono- and oligosaccharides UDP-glucose was removed from the methanol-water phase by elution through Dowex-1 (formiate form, 50 x 4 mm). The eluate was evaporated under reduced pressure to a small volume and submitted to thin-layer chromatography in solvent C (ethyl acetate-acetic acid-water 8:2:2 by vol) with glucose, soluble cellodextrins laminaribiose, laminaritriose and sucrose as references. After development radioactivity was determined as described for UDP-glucose.

2.4.1. Identification of sucrose

A highly radioactive spot, comigrating with sucrose on the thin-layer chromatogram of the soluble mono- and oligosaccharides, was eluted with 2.5 ml water. 1 ml of the extract was added to 1 ml 0.1 M acetate buffer (pH 4.6), containing 0.01% sucrase and 1 ml extract was added to the same buffer, but without sucrase. After 15 min incubation at 37°C the reaction was stopped by heating to 100°C. After elution through Dowex-50 (H^+ form, 50 x 4 mm), to remove Na^+ and protein, the eluates were reduced in volume under reduced pressure and submitted to thin-layer chromatography in solvent C with sucrose and glucose as references. After development 1 cm-bands were scraped off from the chromatograms and eluted with 1.5 ml distilled water.

2.5. Characterization of pellet 1

To investigate the presence of α-1,4-glucosidic polysaccharides pellet 1 was suspended in 0.5 ml 23 mM phosphate buffer (pH 6.8), containing 6 mM NaCl and 1 µg α-amylase. A control pellet was suspended in the same buffer without α-amylase. The suspension was incubated for 1 h at 25°C. The reaction was stopped by addition of 2 ml CH_3OH. The pellet was separated from hydrolysed material by centrifugation for 10 min at 2,500 rpm. The pellet was washed twice with 1 ml 80% CH_3OH. The difference between the radioactivities in the combined supernatants of the α-amylase treated sample and the control sample was taken as a measure for radioactive, α-glucosidic oligo- and polysaccharides.

Possible contamination with radioactive glycoproteins was removed by suspension of pellet 1 in 0.5 ml 0.1 M TRIS-HCl (pH 7.6), containing 2 mM $CaCl_2$ and 0.5 mg pronase. A control pellet was suspended in the same buffer without pronase. The suspensions were incubated for 1 h at 37°C. Insoluble and solubilized material were separated as described for the α-amylase treatment. The pellet, remaining after pronase treatment, is referred to as pellet 2.

Partial acid hydrolysis of pellet 2 and the investigation of this hydrolysate were carried out as described below for partial acid hydrolysis of alkali-insoluble material.

2.6. Characterization of alkali-insoluble material

The alkali-insoluble material used for characterization was obtained by incubation on a threefold scale compared with that described above (2.2.1) with a tenfold higher specific radioactivity of UDP-[^{14}C]glucose. The alkali-insoluble material was hydrolysed with acid or cellulase.

Acid hydrolysis was performed for 3 h with fuming HCl at 25°C. Non-hydrolysed material was removed by centrifugation and washed twice with distilled water. The combined hydrolysate and washes were evaporated to dryness at 36°C to remove HCl. The acid hydrolysate was subjected to thin-layer chromatography in solvent F

(ethyl acetate-acetic acid-water 7.2:2 by vol). Cellotriose, cellobiose, laminaritriose, laminaribiose and glucose were used as reference sugars. From the chromatogram horizontal 0.5 cm-bands (3 cm broad) were scraped off and the radioactive compounds were eluted with 0.5 ml 70% ethanol for 1 h.

Cellulase hydrolysis (10 mg cellulase/ml) was performed for 21 h at 25°C in 0.1 M acetate buffer (pH 4.7) with a crystal of thymol to prevent microbial contamination. Nonhydrolysed material was removed as described for acid hydrolysis. Na^+ and protein were removed from the hydrolysate by means of Dowex-50 ion exchange resin (H^+ form, 50 x 4 mm). The hydrolysate was evaporated to dryness and chromatographed for 60 h on Whatmann no. 1 chromatography paper in butanol-pyridin-water (6:2:3 by vol). The same reference sugars were used as described above. From the chromatogram horizontal 1 cm-bands (4 cm broad) were cut out and the radioactive compounds were eluted for 1 h at 60°C with 0.5 ml 70% ethanol.

2.7. Thin-layer chromatography

Thin-layer chromatography in solvent A: chloroform-methanol-water (62.25:3 by vol) and solvent B: n-propanol-water (7:3 v/v) was performed on Silicagel G plates (Merck no 5721). Thin-layer chromatography in solvent C: ethyl acetate-acetic acid-water (8:2:2 by vol), solvent D: n-butanol-pyridin-water (8:2:1 by vol), solvent E: M ammonium acetate-ethanol (2:5 v/v) and solvent F: ethyl acetate-acetic acid-water (7:2:2 by vol) was performed on Kieselgur plates (Merck no 5738).

2.8. Electron microscopy and electron microscopic autoradiography

For cytochemical studies pollen tubes and membrane pellets were prefixed for 30 min in 6.25% glutaraldehyde/0.1 M phosphate buffer (pH 7.3), containing 0.05 M sucrose, left for one night in the same buffer without glutaraldehyde and postfixed for 30 min in 1% osmiumtetroxide/0.15 M phosphate buffer (pH 7.3). After

dehydration in ethanol the pollen tubes and membrane pellets were flat-embedded in Epon between two glass-slides. Thin sections were poststained with the HIO_4-PTA/CrO_3 staining technique according to Roland et al. (1972) at room temperature or at 38°C. Controls were poststained with lead citrate according to Reynolds (1963).

For comparison of the membrane fractions collected from the 0.5-1.0 M and 0.7-0.9 M sucrose interspaces the membrane pellets, left for one night in 0.1 M phosphate buffer (pH 7.2), containing 4 mM Na_2EDTA were fixed for 1 h in 2% $KMnO_4$ in the same buffer. The fixed pellets were dehydrated with ethanol and embedded in Epon.

For electron microscopic autoradiography the methods described by Vrensen (1970a) were applied. 0.5 µl droplets of the 0.2 ml suspensions (2.2.1) were dried on slides, coated with 0.7% collodion under continuous shaking. The dried material was shadowed with platinum and coated with a thin layer of carbon to prevent interaction with the photographic emulsion. A monolayer of photographic emulsion (Ilford L 4) was applied by means of a semi-automatic coating apparatus (Vrensen, 1970b). After exposure for 2 weeks at 4°C the autoradiographs were developed according to the gold latensification-elon - ascorbic acid procedure (Wisse and Tates, 1968), fixed in 24% sodium thiosulfate and rinsed in water. Copper grids were placed on the specimens. The grids with the autoradiographs were floated on a water surface, picked up with parafilm and air dried.

The thin sections and the autoradiographs were studied in a Philips EM 201 electron microscope.

2.9. Statistical evaluation of the autoradiographs

According to Bachmann et al. (1968) and Salpeter et al. (1969) it seems to be justified to use a theoretical value of resolution in the localization of radioactve material. For statistical evaluation of the autoradiographs the radius of a probability circle, within which 50% of the electrons emitted in the direction of the

emulsion causes a group of silver grains, was calculated to be 100 nm. The features on the autoradiographs were itemized as empty space, amorphous material and microfibrils. The statistical evaluation was carried out as follows: circles with a diameter of 100 nm x photographic magnification were drawn around each group of silver grains on 30 micrographs from areas that were selected at low magnification for the accumulation of amorphous material. Other areas appear to contain no amorphous material or microfibrils and also no accumulation of silver grain groups The (junctional) features within these circles were regarded as probable carriers of radioactivity. To check whether the distribution of silver grain groups merely reflects the distribution of the individual features or whether the silver grain groups indeed accumulate above them, an area measurement was carried out on the same micrographs that were used for the evaluation of grain distribution (Williams, 1969). Transparent plates with 100 randomly distributed circles (radius 100 nm x photographic magnification) were placed on the micrographs and the number of circles above each of the (junctional) features counted.

The significance of the differences (p values) between the silver grain groups/area ratios was determined by a test for a fourfold table (χ^2 or binomial approximation, depending on the marginal totals).

2.10. Analytical procedures

K^+ stimulated Mg^{2+} ATPase, latent IDPase and NADPH cytochrome c reductase, marker enzymes for plasma membranes, Golgi apparatus membranes and endoplasmic reticulum, respectively, were determined according to Hodges and Leonard (1974). Cytochrome c oxydase, a marker enzyme for mitochondria, was determined according to Van Hinsbergh et al. (1978)

Protein was determined according to Lowry et al. (1951) with bovine serum albumin as a standard.

2.11. Radioactivity measurements

In β-glucan synthetase assays and in incorporation studies with isolated lipids radioactivity was measured in 0.5 ml samples after addition of 10 ml scintillation cocktail prepared according to Bray (1960). In the other experiments radioactivity was measured in 1.5 ml samples after addition of 3.5 ml Lumagel as a scintillation cocktail.

2.12. Chemicals

All chemicals were reagent grade. Pronase was obtained from Sigma, sucrase and α-amylase from Merck and cellulase from Schuchardt. Laminaran was obtained from Koch-Light Laboratories, Ltd. and powdered cellulose from Nutritional Biochemicals, Cleveland, Ohio. Lumagel was from Lumac systems AG Basel, Switzerland.

Laminaribiose was prepared by hydrolysis from laminaran (procedure modified from Barry and McCormick, 1962) with boiling H_2SO_4, neutralization with BaO and fractionation of the hydrolysate on Sephadex G-10. Cellotriose was prepared in the same way by hydrolysis from powdered cellulose.

[6,6'-^{3}H]sucrose (3.0 Ci/mmole), UDP-[6-^{3}H]glucose (3.7 Ci/mmole) and UDP-[U-^{14}C]glucose (312 mCi/mmole) were obtained from the Radiochemical Centre, Amersham. GDP-[U-^{14}C]glucose (83 mCi/mmole) was obtained from ICN Isotope and Nuclear Division, Irvine, California.

Cytochemical staining and marker enzyme activities of membranes of germinating Petunia pollen

3.1. Introduction

The periodic acid-phosphotungstate/chromic acid (HIO_4-PTA/CrO_3) staining technique according to Roland et al. (1972) is widely used as a selective staining procedure for plasma membranes of plant cells, also in fractionation studies (Koehler at al., 1976; Leonard and Van Der Woude, 1976, Nagahashi et al., 1978). Thom et al. (1975) and Quail and Hughes (1977) found that membranes of prolamellar bodies, lipid droplets and ribosomes are also stained. Oligo- and polysaccharides appear to be the reacting molecules (Pease, 1970) However, the exact nature of the stain is still unknown (Roland, 1978).

Pollen tubes show a large accumulation of Golgi vesicles in the apical zone (Sassen, 1964, Van Der Woude et al., 1971; Cresti et al., 1977). These Golgi vesicles undergo a maturing process in the course of their migration from the dictyosomes to the apical zone. During this process the membrane of the vesicles changes in biochemical composition in order to facilitate a fusion with the plasma membrane (Roland, 1973; Montezinos and Brown, 1976). Cell wall precursors are synthesized inside the Golgi vesicles which are released into the cell wall after this fusion.

In this study the maturing process of Golgi vesicles was investigated in germinating *Petunia* pollen with the HIO_4-PTA/CrO_3 staining technique. In addition to this technique marker enzyme assays were employed to characterize membrane fractions isolated from these germinating pollen

3.2.1. In situ studies

Fig. 3.1. shows electron micrographs of pollen tubes of *Petunia hybrida* after poststaining with lead citrate and with HIO_4-PTA/CrO_3, respectively. Golgi vesicles, which are abundant in the apical zone of the pollen tube, appear electron-transparent after lead citrate poststaining and electron-dense after HIO_4-PTA/CrO_3 poststaining (Figs. 3.1 and 3.2). With both staining procedures lipid droplets, the plasma membrane and the cell wall of the pollen tube are also stained (Figs. 3.1 and 3.4). An electron-transparent zone can be observed in the cell wall of the apical zone (Fig. 3 4) Dictyosomes (Fig. 3 4), mitochondria and endoplasmic reticulum do not stain with HIO_4-PTA/CrO_3.

Fig. 3.2 shows micrographs of two subsequent sections of the same pollen tube tip, poststained with lead citrate and HIO_4-PTA/CrO_3, respectively. In this way identical vesicles, having a diameter of about 0.3 µm, can be compared with respect to their reaction to both staining techniques. After staining with lead citrate the vesicle content and membrane appear less electron-dense than with HIO_4-PTA/CrO_3.

Approaching the apical zone of the pollen tube the staining of the Golgi vesicles with HIO_4-PTA/CrO_3 becomes more intense (Fig. 3.3). In the apical zone the Golgi vesicles fuse with each other and with the plasma membrane (Figs. 3.3 and 3.4) and release their content into the cell wall.

The HIO_4-PTA/CrO_3 staining *in situ* was carried out at room temperature. At 38°C the membranes became more intensely stained, but the specificity remained

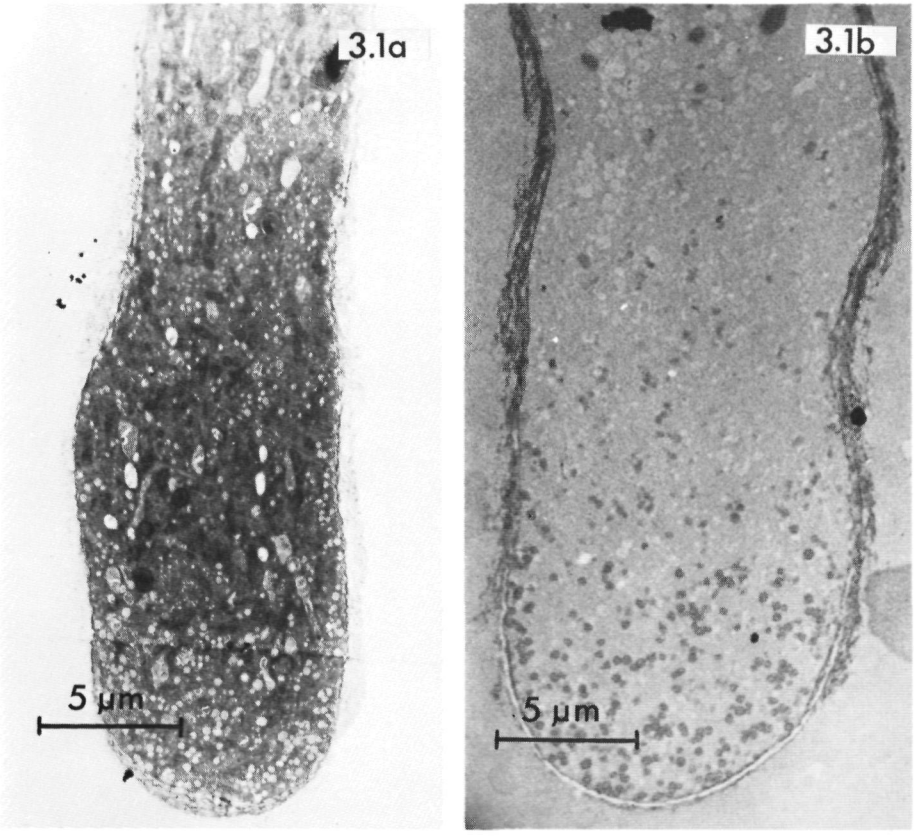
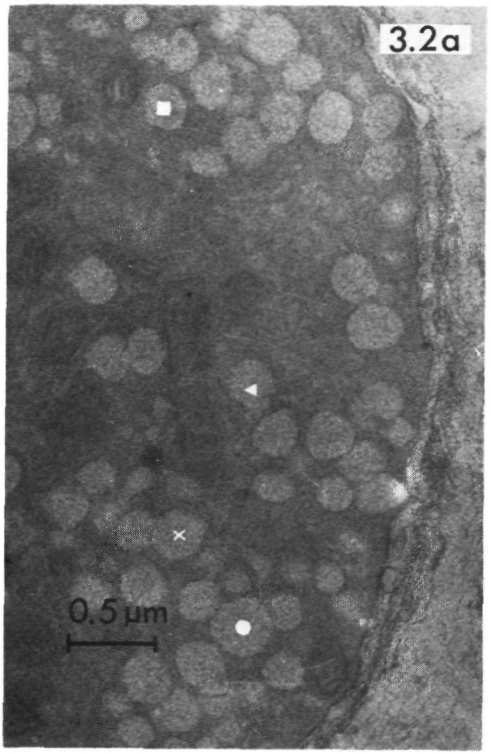


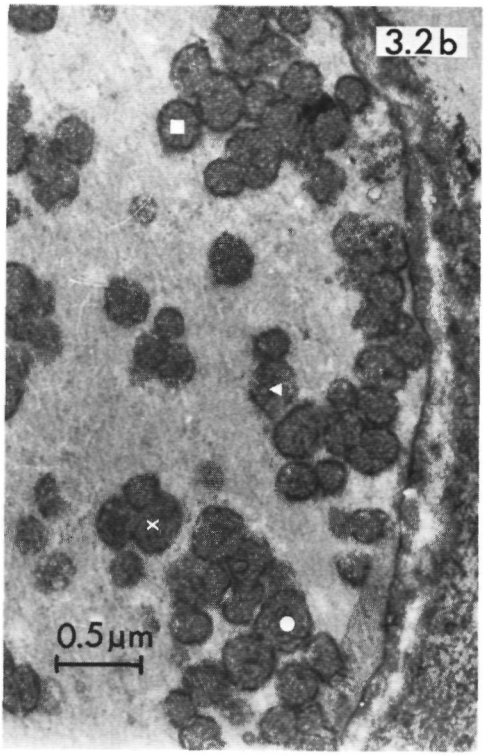
Fig. 3.1. Electron micrographs from *Petunia* pollen tubes poststained with a) lead citrate and b) HIO_4-PTA/CrO_3. Magnification: 3,700 x

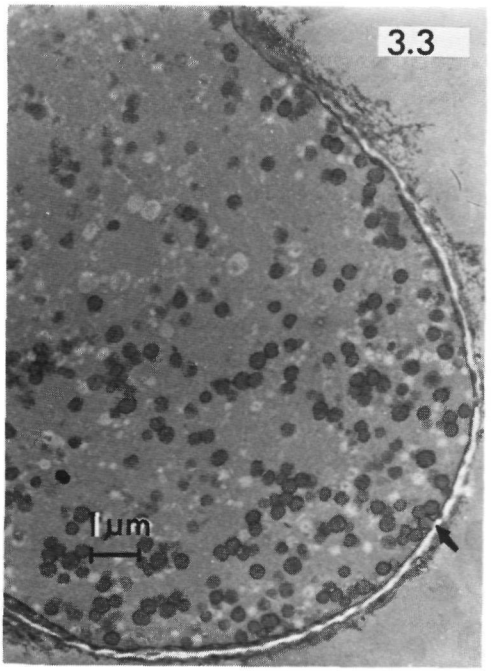
Fig. 3.2. Electron micrographs from two subsequent sections of the same *Petunia* pollen tube tip poststained with a) lead citrate and b) HIO_4-PTA/CrO_3. Identical Golgi vesicles are indicated by corresponding symbols. M = mitochondrion. Magnification: 22,500 x

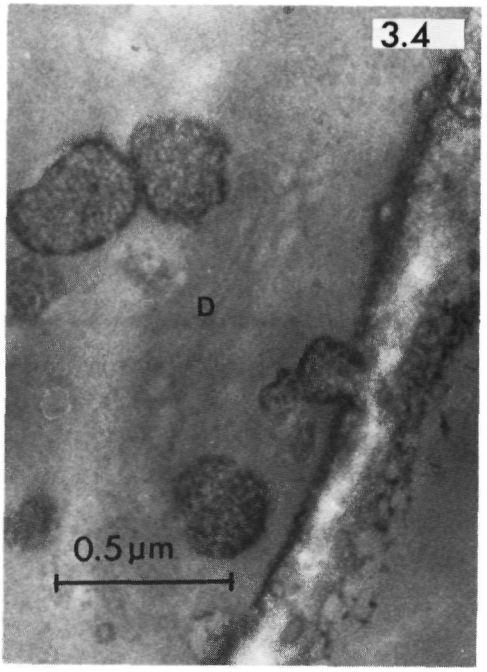
Fig. 3.3. Electron micrographs from a *Petunia* pollen tube tip poststained with HIO_4-PTA/CrO_3. The arrow head points to Golgi vesicles fusing with each other and with the plasma membrane. Magnification: 6,000 x

Fig. 3.4. Detail of Golgi vesicles and plasma membrane after staining with HIO_4-PTA/CrO_3. One of the Golgi vesicles is fusing with the plasma membrane. Note the dictyosome that does not contrast with this staining. Magnification: 45,000 x


3.2a
0.5 µm


3.2b
0.5µm


3.3
1µm


3.4
D
0.5µm

3.2.2. Characterization of the membrane fractions

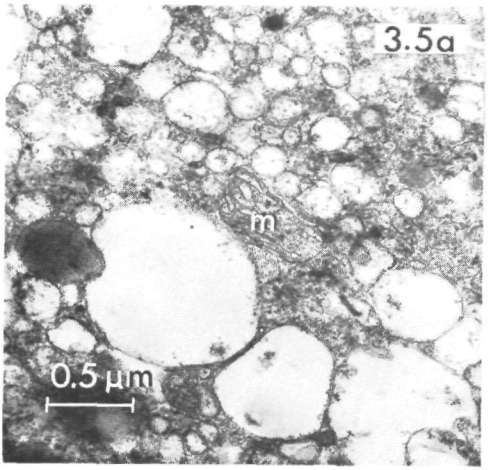
3.2.2a HIO_4-PTA/CrO_3 staining

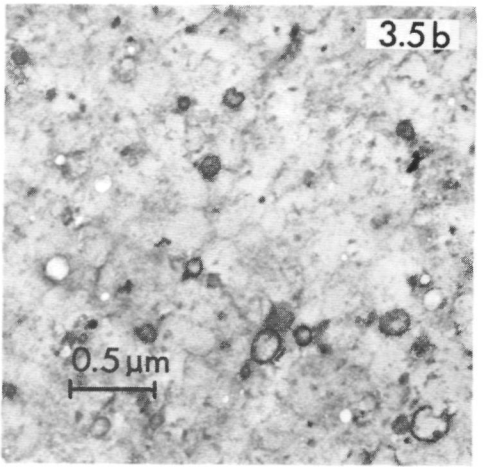
Figs. 3.5-3.7 show the rough membrane fraction and the membrane fractions collected at the 0.5-1.0 M and the 1.0-1.5 M sucrose interspaces. These fractions were poststained with lead citrate or with HIO_4-PTA/CrO_3 at 38°C. Upon staining with HIO_4-PTA/CrO_3 at room temperature none of these fractions showed contrasting membranes. If glutaraldehyde was added before homogenization of the germinating pollen some stained vesicles were observed after staining at room temperature. The material collected at the 0.3-0.5 M sucrose interspace, containing small HIO_4-PTA/CrO_3-negative vesicles and lipid droplets embedded in amorphous material, is not documented here. The material collected at the 1.5-2.0 M sucrose interspace is also not documented, because it looked similar to that collected at the 1.0-1.5 M sucrose interspace.

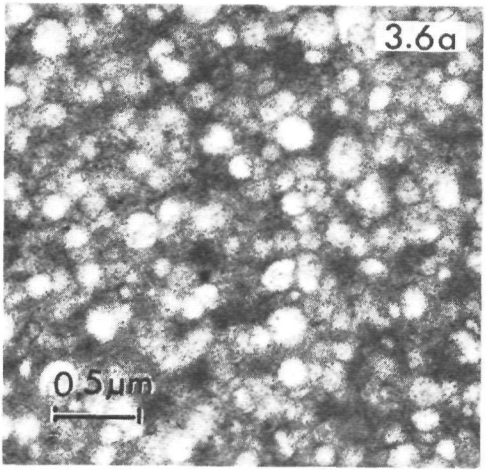
After lead citrate poststaining the rough membrane fraction (Fig 3.5a) shows intact mitochondria and various types of membranes. After staining with HIO_4-PTA/CrO_3 only some of the membrane vesicles were contrasting (Fig. 3.5b). A few also have an electron-dense content. These have a diameter that was slightly smaller (about 0.2 µm) than the Golgi vesicles in the pollen tube.

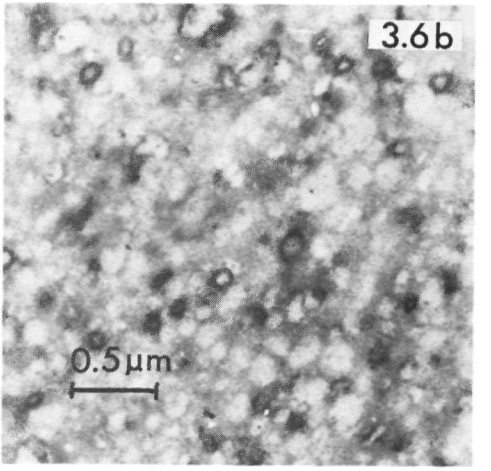
In the membrane fraction collected at the 0.5-1.0 M sucrose interspace electron-transparent vesicles with a diameter of about 0.3 µm predominate after lead citrate poststaining (Fig. 3.6a). These do not stain with HIO_4-PTA/CrO_3 (Fig. 3.6b). The vesicles with electron-dense content and membrane are more abundant in this fraction than in the rough membrane fraction and have a diameter of about 0.2 µm. Also some electron-transparent vesicles with an electron-dense membrane are observed with the latter stain.

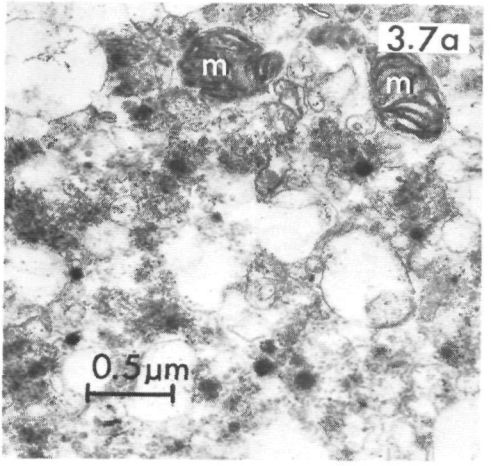
The material collected at the 1.0-1.5 M sucrose interspace contains mitochondria and various types of membranes (Fig. 3.7a). After staining with HIO_4-PTA/CrO_3 some vesicles have an electron-dense membrane and content (Fig. 3.7b). Some have only an electron-dense membrane, while mitochondria and other membranes do not stain at all.

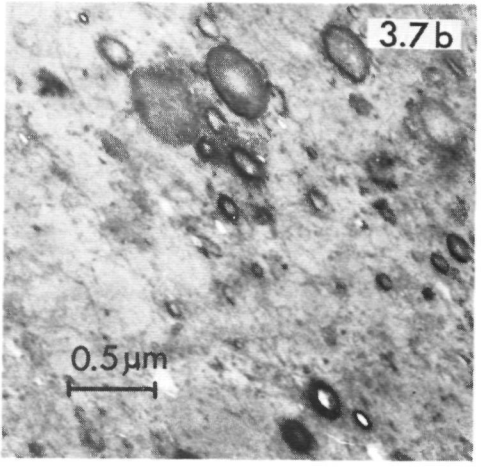

3.5a
m
0.5 µm


3.5b
0.5 µm


3.6a
0.5 µm


3.6b
0.5 µm


3.7a
m
m
0.5 µm


3.7b
0.5 µm

3.2.2.b Activities of marker enzymes

Table 3.2. shows marker enzyme activities of the rough membrane fraction and the fractions collected at the four sucrose interspaces. The material collected at the 0.3-0.5 M sucrose interspace showed hardly any of these enzyme activities, indicating that this fraction contains proteins that originate from cytoplasmic protein. The material collected at the 0.5-1.0 M sucrose interspace appears to be enriched in latent IDPase and K^+ stimulated Mg^{2+} ATPase in comparison with the rough membrane fraction, but

Table 3.1. Marker enzyme activities in the rough membrane fraction and the four different membrane fractions collected from the discontinuous sucrose gradient (2.1) The activities are expressed in µmoles of product converted per mg protein per hour

Membrane fraction	K^+ stimulated Mg^{2+} ATPase	Latent IDPase	NADPH cyt c reductase	Cyt c oxydase
Rough membrane fraction	2.2	9.6	16.8	181
0.3-0.5 M	0.6	1.0	1.2	28
0.5-1.0 M	3.8	13.8	6.0	158
1.0-1.5 M	4.6	8.8	21.6	341
1.5-2.0 M	5.4	2.6	26.4	327

Figs. 3.5-3.7. Electron micrographs of the rough membrane fraction (Figs. 3.5) and of the membrane fractions collected at the 0.5-1.0 M (Figs. 3.6) and the 1.0-1.5 M (Figs. 3.7) sucrose interspaces (2.1). Figs. 3.5a, 3.6a and 3.7a show poststaining with lead citrate, Figs. 3.5b, 3.6b and 3.7b with HIO_4-PTA/CrO_3. M = mitochondrion. Magnification. 22,500 x

also NADPH cytochrome c reductase and cyrochrome c oxydase are present in this fraction. K^+ stimulated Mg^{2+} ATPase, NADPH cytochrome c reductase and cytochrome c oxydase accumulate in the fractions collected at the 1 0-1.5 M and 1.5-2.0 M sucrose interspaces to about equal levels. Latent IDPase activity in these two fractions is lower than in the rough membrane fraction and the fraction collected at the 0.5-1.0 M sucrose interspace.

3.3. Discussion

The results support existing theories concerning the role of Golgi vesicles in the synthesis of plant cell walls. According to these theories cell wall precursors are synthesized inside the Golgi vesicles during their migration from the dictyosomes to the plasma membrane. After the fusion of the Golgi vesicle membrane with the plasma membrane the cell wall precursors may be incorporated into the cell wall (Sassen, 1964, Van Der Woude et al., 1971; Cresti et al., 1977). The increase in staining intensity of the content of the Golgi vesicles with HIO_4-PTA/CrO_3 near the apical zone is indicative for an accumulation of oligo- and polysaccharides which might be cell wall precursors The increase in contrast of the membranes of the Golgi vesicles during their migration from the dictyosomes to the plasma membrane suggests changes in their chemical composition which facilitate the fusion process. Roland (1973) described similar changes with the HIO_4-PTA/CrO_3 staining of Golgi vesicle membranes in pea cells

It is questionable whether membranes of mature Golgi vesicles should be regarded as Golgi vesicle membranes or as (future) plasma membranes. The HIO_4-PTA/CrO_3 staining is not specific for plasma membranes as Roland stated (Roland et al., 1972, Roland, 1978), since also other cell constituents are stained. This has been reported earlier by Thom et al. (1975) and Quail and Hughes (1977).

The fact that membranes lost their staining capacity for HIO_4-PTA/CrO_3 after fractionation has been observed earlier with plasma membranes from corn cells by Leonard and Van Der Woude

(1976). They assumed that the used Ficoll caused this anomalous effect. This effect was abolished by staining at 38°C. Also in our studies staining at 38°C improved the contrast of fractionated membranes. The loss of staining capacity in our experiments might be due to glycosidase activity on membrane-bound oligosaccharides, since it could be prevented by the addition of glutaraldehyde before homogenization of the germinating pollen.

The abundance of vesicles in the membrane fraction collected at the 0.5-1.0 M sucrose interspace with HIO_4-PTA/CrO_3-positive content and membrane points to the accumulation of Golgi vesicles. The smaller diameter of the fractionated vesicles might be due to shrinkage. The higher value for latent IDPase in this membrane fraction in comparison with the other fractions also indicates an accumulation of Golgi membranes (Hodges and Leonard, 1974; Bowles and Kauss, 1976; Morré et al., 1977). The presence of electron-transparent vesicles with electron-dense membrane after HIO_4-PTA/CrO_3 and of the K^+ stimulated Mg^{2+} ATPase also suggests the presence of plasma membranes (Leonard and Van Der Woude, 1976, Nagahashi et al., 1978). However, the specificity of the latter enzyme as a marker for plasma membranes is doubted (Leigh et al., 1975; Hendriks, 1977, 1978). Assuming a membrane flow from dictyosomes via Golgi vesicles to the plasma membrane (Goff, 1973) it is questionable anyway whether marker enzymes can differentiate between these membranes. NADPH cytochrome c reductase and cytochrome c oxydase activities, although relatively low, show the presence of endoplasmic reticulum and mitochondrial membranes, respectively (Hodges and Leonard, 1974), in this fraction.

Marker enzyme activities in the membrane fractions from the 1.0-1.5 M and the 1.5-2.0 M sucrose interspaces indicate that plasma membranes, endoplasmic reticulum and mitochondrial membranes are present in higher concentration and Golgi membranes in lower concentration than in the membrane fraction collected at the 0.5-1.0 M sucrose interspace. The electron microscopical results support the similarity in composition of these fractions. The electron-transparent vesicles with electron-dense membrane after HIO_4-PTA/CrO_3 staining probably represent plasma membranes.

Characterization of β-glucan synthetase activity in the membrane fraction

4.1. Introduction

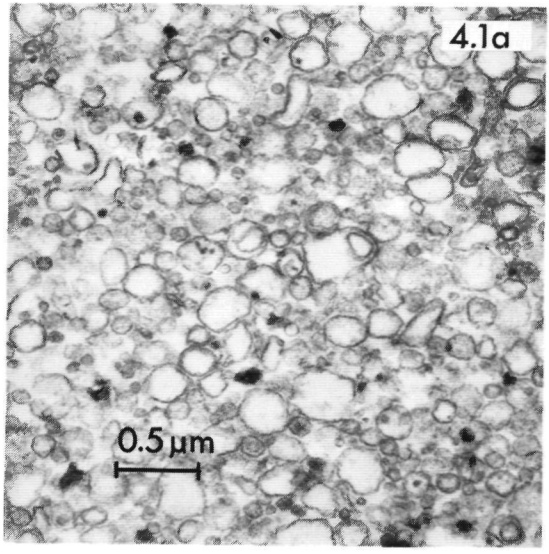
Many investigators have shown β-glucan synthetase activity (E.C. 2.4.1.12 and 2.4.1.34) in isolated membrane systems from *Acetobacter xylinum* (Glaser, 1958; Cooper and Manley, 1975a, 1975b), *Acanthamoeba* (Potter and Weisman, 1971) and higher plants (Ray et al., 1969; Van Der Woude et al., 1974). The plasma membranes were found to be the main site of cellulose synthesizing enzymes in higher plants, but Golgi vesicles have also been shown to contain cellulose synthetase activity (Ray et al., 1969; Van Der Woude et al., 1974).

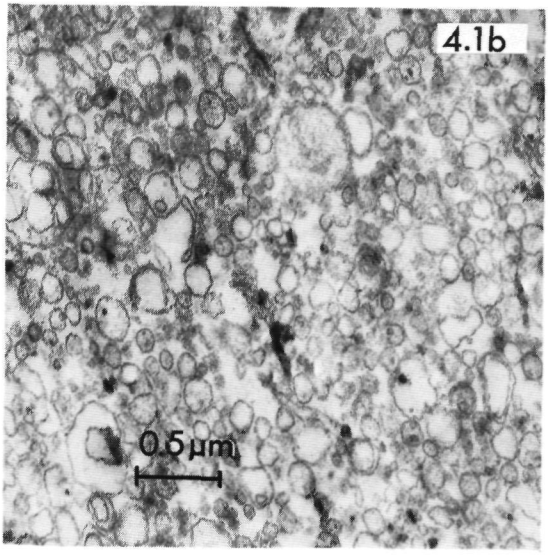
Particulate enzymes of bacteria (Cooper and Manley, 1975a) and *Acanthamoeba* (Potter and Weisman, 1971) use mainly UDP-glucose as precursor for the in vitro synthesis of β-glucans; particulate enzymes of higher plants generally use GDP-glucose as well as UDP-glucose for the production of β-glucosidic linked polysaccharides.

The presence of cellulose in Golgi vesicles of *Pleurochrysis scherffelii* was reported by Brown et al. (1969, 1970,1973). Engels (1973, 1974a, 1974b) obtained evidence for the presence of cellulose in a membrane fraction from germinating *Petunia* pollen by means of X-ray diffraction. On morphological basis he identified this fraction as Golgi vesicles.

This chapter concerns the presence and characterization of a particulate β-glucan synthetase activity in a membrane fraction from germinating *Petunia* pollen, which is morphologically similar to that used by Engels (1973, 1974a, 1974b).

4.2.1. Morphological comparison of the fractions from the 0.5-1.0 M and 0.7-0.9 M sucrose interspaces





Figs. 4.1a and 4.1b show the membrane fractions of *Petunia* pollen tubes obtained from the 0.5-1.0 M interface of the first centrifugation step and from the 0.7-0.9 M interface of the second centrifugation step, respectively, in the isolation procedure according to Engels (1973).

Comparison of the electron micrographs suggests that the 0.7-0.9 M fraction is not much purer than the 0.5-1.0 M fraction. Both show vesicle structures of about 0.5 µm with electron transparent

Figs. 4.1a and 4.1b Electron micrographs of the membrane fractions at the 0.5-1.0 M interface of the first sucrose gradient (a) and at the 0.7-0.9 M interface of the second sucrose gradient (b). Magnification 22,500 x

content.

The loss in protein in the second centrifugation step was 70-80% from the amount present in the 0.5-1.0 M fraction. Since the second centrifugation step did not improve the purity, but caused a loss of much activity, we omitted this step and used the 0.5-1.0 M fraction in further studies. It will be referred to as "the membrane fraction".

4.2.2. β-Glucan synthetase activity of the four different sucrose gradient fractions

All fractions of the sucrose gradient except the 0.3-0.5 M fraction incorporated [^{14}C]glucose from UDP-[^{14}C]glucose into lipid-soluble, alkali-soluble and alkali-insoluble material. The radioactivity incorporated into alkali-insoluble glucan by the 1.0-1.5 M and the 1.5-2.0 M fractions was higher than by the 0.5-1.0 M fraction (Table 4.1).

Table 4.1. β-Glucan synthetase activities of the four different fractions of the sucrose gradient. The radioactivity is given as pmoles of glucose incorporated per mg protein into alkali-insoluble [^{14}C]glucan. The sucrose gradient fractions were incubated with 5.8 nmoles UDP-[^{14}C]glucose (7 x 10^4 dpm) or 5.8 nmoles GDP-[^{14}C]glucose (7 x 10^5 dpm) for 15 min at 25°C (2.2.1).

	Glucosyl donor	
	UDP-[^{14}C]glucose	GDP-[^{14}C]glucose
0.3-0.5 M fraction	5.2	0.6
0.5-1.0 M fraction	63.3	0.5
1.0-1.5 M fraction	99.0	0.6
1.5-2.0 M fraction	115.0	0.5

None of the four sucrose fractions incorporated a significant

amount of radioctivity from GDP-[^{14}C]glucose into lipid-soluble, alkali-soluble or alkali-insoluble material, even though the specific radioactivity of the GDP-[^{14}C]glucose was ten times higher than that of the UDP-[^{14}C]glucose solution. Thin-layer chromatography showed that intact GDP-[^{14}C]glucose did not compose during incubation.

4.2.3. Optimum conditions for β-glucan synthetase activity of the membrane fraction

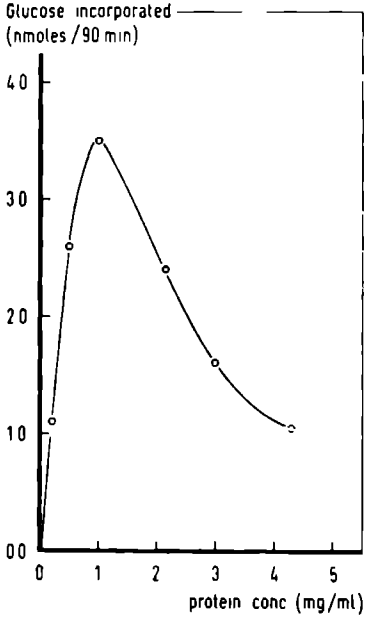


Fig. 4.2. Effect of the protein concentration of membrane fraction in the incubation medium on the incorporation of UDP-[^{14}C]glucose into alkali-insoluble material

An optimum β-glucan synthetase activity is reached at 1 mg protein per ml reaction medium (Fig. 4.2). An approximately linear relationship between β-glucan synthetase activity and the concentration of membraneous protein is observed up to 1 mg protein per ml. Above this concentration the amount of alkali-insoluble material, formed during incubation with UDP-[^{14}C]glucose, decreases with increasing protein concentration.

The time course of *in vitro* synthesis of alkali-insoluble material shows an approximately linear increase with time (Fig 4.3). The slight decrease in reaction rate after 60 min might be due to a decrease in substrate (UDP-[^{14}C]glucose) concentration and/or denaturation of the enzyme. For practical reasons the reaction time was kept to 90 min.

The decrease in synthesis rate of alkali-soluble and lipid-soluble material begins earlier (Fig. 4.3).

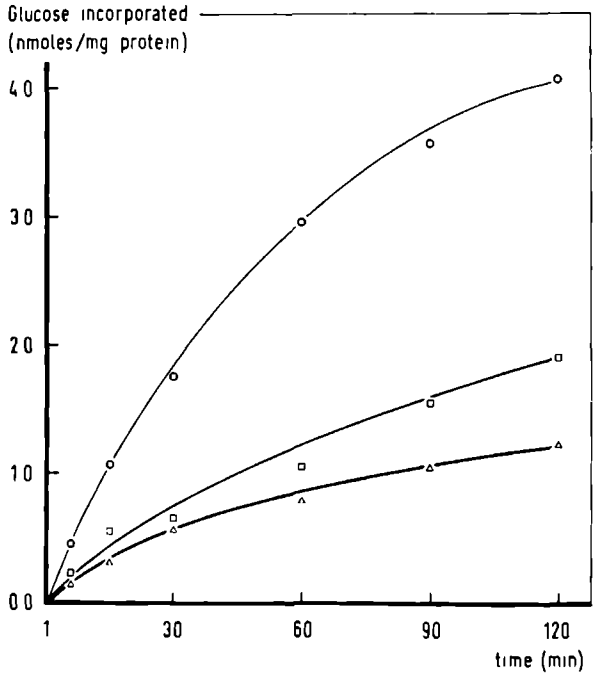


Fig. 4.3. Time course of the incorporation of UDP-[^{14}C] glucose into lipid-soluble (□-□), alkali-soluble (Δ-Δ) and alkali-insoluble material (o-o) by the membrane fraction

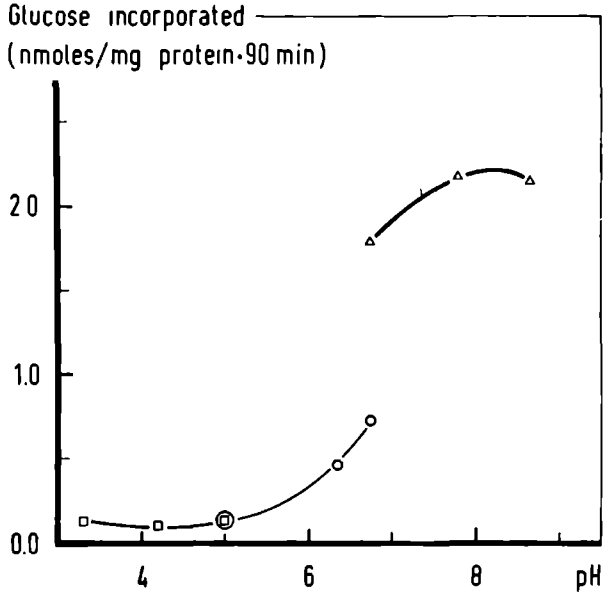


Fig. 4.4. pH-dependence of and effect of the incubation buffer on the incorporation of UDP-[^{14}C] glucose into alkali-insoluble material. □-□ = acetate buffer, o-o = phosphate buffer, Δ-Δ = TRIS-HCl buffer

After 90 min the radioactivity incorporated into the lipid-soluble and the alkali-soluble material was about 40% and 30%, respectively, of that into the alkali-insoluble material.

Fig. 4.4 shows the pH dependence of the β-glucan synthetase activity of the membrane fraction. The optimum pH for the system appears to be about 8. In TRIS-HCl buffer β-glucan synthetase activity was 2.5 times that observed in phosphate buffer of the same pH.

The optimum temperature for β-glucan synthetase activity of the membrane fraction is dependent on the incubation time (Fig. 4.5). After 10 min the maximum amount of alkali-insoluble material is formed at 30°C. After 90 min denaturation may become predominant and the optimum temperature shifts to 25°C.

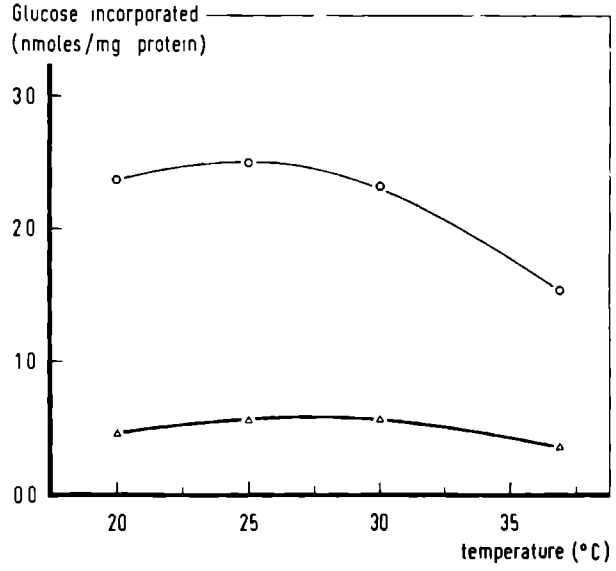


Fig. 4.5. Effect of temperature and incubation time on the incorporation of UDP-[^{14}C]glucose into alkali-insoluble material. Times of incubation were 10 min (Δ-Δ) and 90 min (o-o)

4.2.4. Characterization of the alkali-insoluble material

Table 4.2 shows the proportion of radioactivity found in the cellulase and the acid hydrolysates of the alkali-insoluble material. The material at the origin region of the chromatogram consists of

Table 4.2. Composition of the partial acid and partial cellulase hydrolysates of the alkali-insoluble material formed by incubation of the membrane fraction with UDP-[^{14}C]glucose (2.6). The composition is given in percentages of the total radioactivity present in the hydrolysates

	Acid hydrolysate	Cellulase hydrolysate
Material at the origin	79.4	
Cellotriose	7.7	}7.0
Cellobiose	5.9	25.5
Laminaritriose	0.5	26.5
Laminaribiose	0.3	19.5
Glucose	6.2	21.5

oligosaccharides that contain between 4 and 8 sugar residues. In the cellulase hydrolysate cellotriose was not distinguishable from the material at the origin region of the chromatogram.

The results obtained from the partial acid hydrolysis suggest that the alkali-insoluble material consists mainly of β-1,4-glucosidic bonds. Hydrolysis with the crude cellulase preparation, however, shows that also β-1,3-glucosidic linkages are present in the newly-synthesized alkali-insoluble [^{14}C]glucan.

4.3. Discussion

A β-glucan synthetase, which catalyses the incorporation of glucose from UDP-glucose and not from GDP-glucose into β-glucans is rarely found in higher plants. In addition to the enzyme described here from germinating *Petunia* pollen, only two other membrane systems have been isolated from higher plants, that are incapable of synthesizing β-glucans from GDP-glucose. Both systems, one from *Lilium longiflorum* pollen (Southworth and Dickinson, 1975)

and one from cotton fibers (Delmer et al., 1976), synthesize only β-1,3-glucans.

An optimum pH of about 8 was found for β-glucan synthetase activities from *Lupinus albus* (Larsen and Brummond, 1974) and from oat seedlings (Pinsky and Ordin, 1969). The higher activity in TRIS-HCl buffer compared to phosphate buffer has also been reported for β-glucan synthetase activities from other plant species (Pinsky and Ordin, 1969).

The chromatographic study of the partial acid hydrolysate of the alkali-insoluble material suggests an almost completely β-1,4-glucosidic bound polysaccharide, Partial enzymic hydrolysis, however, proves that also β-1,3-glucosidic links are present. β-1,3-Glucosidic links are probably much more sensitive to acidic hydrolysis than β-1,4-glucosidic ones, so that the latter type of bond is predominantly demonstrated after partial acid hydrolysis. As Péaud-Lenoël and Axélos (1971) have shown,one cannot deduce a purely β-1,4- glucosidic bound polysaccharide from the absence of laminaribiose and laminaritriose in the chromatogram of partial acid hydrolysates.

The possible role of lipid intermediates in the synthesis of β-glucans by the membrane fraction

5.1. Introduction

The role of polyprenol (pyro)phosphate sugars as intermediates in the synthesis of glycoproteins and polysaccharides from nucleoside diphosphate sugars is established in a wide variety of microorganisms, plants and animals. Recent reviews (Waechter and Lennarz, 1976; Hemming, 1977) refer to more than 100 reports about this subject.

Polyprenol (pyro)phosphate sugars function as intermediates in the synthesis of bacterial (Sutherland, 1975) and yeast (Jung and Tanner, 1973) cell wall polysaccharides. Recently Hopp et al. (1978) obtained more conclusive evidence with particulate preparations from *Prototheca zopfii* for a reaction pathway from UDP-glucose via polyprenol monophosphate glucose, polyprenol pyrophosphate glycosides and glycoprotein to cellulose, the most abundant cell wall polysaccharide.

In this chapter the role of glucolipids as intermediates for β-glucan synthesis by the membrane fraction from Petunia pollen tubes is investigated.

5.2. Results

5.2.1. Characterization of lipids

Thin-layer chromatography of the crude lipid preparation in solvent A shows 5 labelled compounds (Fig. 5.1). One of them remains at the origin of the chromatogram, the others have

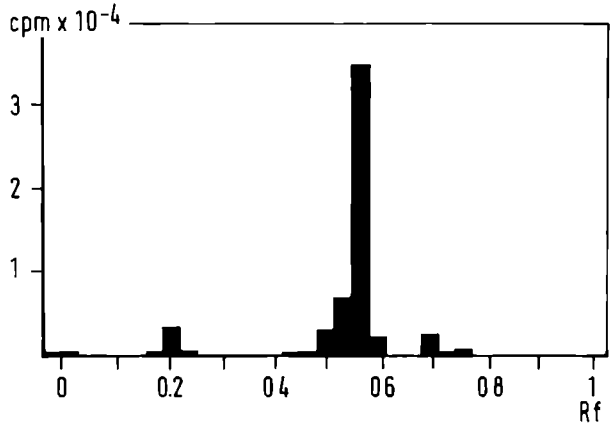


Fig. 5.1. Thin-layer chromatography of the crude lipid preparation in solvent A

Table 5.1. Radioactivity (in dpm and percentage) in substrate (UDP-glucose) and reaction products after 30 min incubation of the membrane fraction (containing 0.2 mg protein) with 5 µCi UDP-[^{3}H]glucose (2.2.2). n.d. = not determined. The values are the mean of three experiments

Compound	Radioactivity	
	dpm	%
UDP-glucose	3,830,000	52.9
Polyprenol monophosphate glucose	23,800	0.3
Sterol glucosides	225,000	3.1
Glucose	298,000	4.1
Sucrose	1,647,000	22.7
Cellobiose	n.d.	n.d.
Cellotriose	34,000	0.5
Cellotetraose + cellopentaose	42,500	0.6
Pellet 2 (insoluble β-glucans)	694,000	9.6

Rf values of 0 2, 0.57, 0.70 and 0 77.

At DEAE cellulose chromatography the material at the origin is not eluted from the column. The material with Rf values higher than 0.5 eluted from this column with $CHCl_3$-CH_3OH (2 1 v/v) as the neutral lipid fraction These lipids are alkali-stable, indicating that they are sterol glucosides

Subsequent elution of the column was carried out with 99% CH_3OH with increasing ammonium acetate concentrations It was shown that the radioactive compounds were released exclusively by 99% CH_3OH with 0.01 M ammonium acetate. Thin-layer chromatography of this fraction in solvent A shows an Rf value of 0.2. In solvent B the same compound has an Rf value of 0.75. Mild acid hydrolysis of this fraction shows, that it is acid-labile (80 % of the radioactivity is released from the lipid) Thin-layer chromatography of the hydrolysate in solvent D shows glucose as the only labelled compound

5.2.2. Characterization of compounds in the methanol-water phase

After 30 min incubation about 45 % of the UDP-glucose, added to the incubation medium, was converted into reaction products (Table 5 1.). About 23 % is incorporated into sucrose, identified by its digestibility with sucrase. Sucrose synthetase activity was inhibited for about 30 % by addition of 2 mM NADPH, which did not affect β-glucan synthetase activity. When the membrane fraction was incubated with [^{3}H]sucrose no incorporation of radioactivity into UDP-glucose or β-glucans was observed. Radioactivity in cellobiose could not be quantified, because it is partially masked by sucrose. Radioactive laminaribiose or laminaritriose could not be detected. Glucose, cellobiose, cellotriose, cellotetraose and possibly cellopentaose are minor components in the methanol-water phase

5.2.3. Characterization of pellet 1

α-Glucans were not detected in pellet 1 with α-amylase treatment. Five percent of the labelled material was digested by

Table 5.2. Compostıon of the partıal acid hydrolystates of the alkalı-insoluble materıal (Table 4.2) and of pellet 2 (methanol ınsoluble β-glucans) (2.6). The compositıon is gıven ın percentages of the total radioactıvity

	Alkali-insoluble material	Pellet 2 (β-glucans)
Material at the origin	79.7	73.6
Cellotriose	7.7	11.6
Cellobiose	5.9	2.8
Glucose	6.2	12.0

pronase, indicatıng the presence of radıoactive glycoproteins. After partıal acid hydrolysis of the pronase-treated pellet (pellet 2) thin-layer chromatography showed glucose, cellobiose, cellotrıose and higher cellodextrıns, the latter remaıning at the orıgın (Table 5.2). The composition of thıs hydrolysate resembled that of the alkali-insoluble materıal, synthesızed ın a similar reactıon (chapter 4). Pellet 2 seems to consıst of methanol-ınsoluble β-glucans.

5.3.4. *Incorporation studies with isolated lipids*

When the radioactıve, Triton X-100-solubilized crude lıpıd preparation or polar lıpıds were used as substrate for β-glucan synthesıs, ıt was ımpossible to detect any enzymatıc transfer of radıoactivıty to alkalı-ınsoluble materıal (Fig. 5.2). Addıtion of 20 mM $MgCl_2$, 20 $MnCl_2$, 10 mM $MgCl_2$ plus 10 mM $MnCl_2$ or 2 mM unlabelled UDP-glucose at 0.01 % Trıton X-100 had no effect. With UDP-[^{14}C]glucose as substrate the ıncorporatıon of radıoactivıty ınto alkalı-ınsoluble materıal

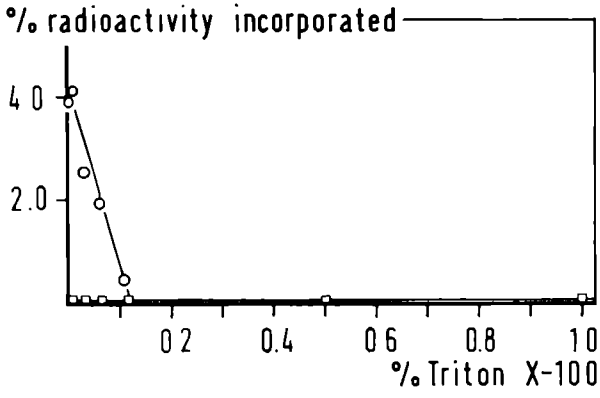


Fig. 5.2. Effect of the Triton X-100 concentration in the incubation medium on the incorporation of radioactivity from UDP-[^{14}C]glucose (o-o) and from crude lipid preparation or polar lipids (□-□) into alkali-insoluble material (2.2.2.)

(Fig. 5.2) decreased linearly from 4 to 0% when the Triton X-100 concentration was increased from 0 to 0.12%.

5.2.3. Kinetic experiments

Lineweaver-Burk plots of the incorporation of UDP-glucose into pellet 2 (methanol-insoluble β-glucans) and sterol glucosides are linear (Fig. 5.3). For the polar lipids a slightly curved line is observed, probably due to a biphasic kinetic. In the concentration range from 2 to 100 µM UDP-glucose the initial incorporation rates for polar lipids and sterol glucosides are lower than that for pellet 2.

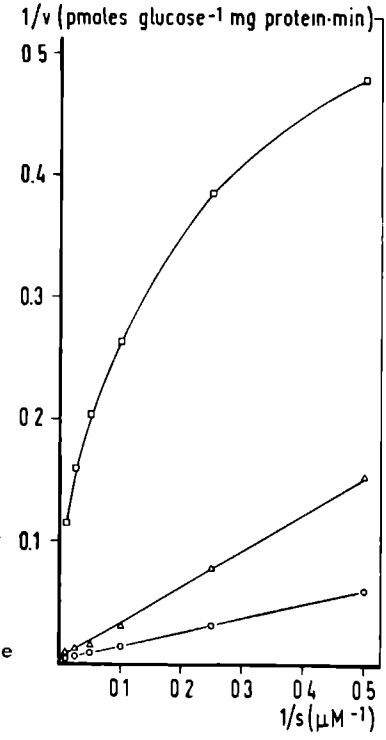


Fig. 5.3. Lineweaver-Burk plots of the synthesis of pellet 2 (methanol-insoluble β-glucans)(o-o), sterol glucosides (Δ-Δ), and polar lipids (□-□) with UDP-[^{14}C]glucose as substrate (2.2.2)

Compound	30 min	31 min			32 min			35 min		
	^{3}H	^{3}H	^{14}C	$^{3}H/^{14}C$ ratio	^{3}H	^{14}C	$^{3}H/^{14}C$ ratio	^{3}H	^{14}C	$^{3}H/^{14}C$ ratio
UDP-glucose	3,692,000	3,384,000	1,748,000	1.9	3,268,000	1,542,000	2.1	2,955,000	1,518,000	1.9
Polyprenol monophosphate glucose	24,000	22,000	67	328	19,650	140	140	17,340	264	66
Sterol glucosides	225,000	230,800	1,620	142	239,600	3,100	77	244,000	7,200	34
Glucose	304,000	295,700	610	485	287,700	1,100	261	283,900	2,790	102
Sucrose	-	-	-	130	-	-	66	-	-	26
Cellobiose	-	-	-	84	-	-	34	-	-	20
Cellotriose	-	-	-	180	-	-	106	-	-	52
Cellotetraose + cellopentaose	-	-	-	112	-	-	74	-	-	26
Pellet 2 (insoluble β-glucans)	723,000	740,000	6,400	116	772,000	11,700	66	779,000	31,000	25

5.2.6. Double-labelling experiments

Table 5.3 shows the results of one representative duplicate double-labelling experiment out of a series of three. It gives the radioactivity in dpm and the $^{3}H/^{14}C$ ratios found in the substrate (UDP-glucose) and the reaction products at 1, 2 and 5 min after the addition of UDP-[^{14}C]glucose to an incubation medium, containing the membrane fraction, which was incubated for 30 min with UDP-[^{3}H]glucose. Total ^{14}C-radioactivities could not be accurately determined in the poorly labelled oligosaccharides because of partial overlapping of the spots. $^{3}H/^{14}C$ ratios in these compounds were determined in the peak areas of the respective spots, which were not contaminated by other oligosaccharides.

After corresponding incubation periods glucose always has the highest $^{3}H/^{14}C$ ratios of all compounds. The $^{3}H/^{14}C$ ratios in polar lipids and sterol glucosides are higher than those for sucrose, cellobiose, cellotetraose + cellopentaose and pellet 2. The $^{3}H/^{14}C$ ratios in cellotriose were between those for polar lipids and sterol glucosides.

During the double-labelling period the increase in ^{14}C-radioactivity is accompanied in most reaction products by an increase in ^{3}H-radioactivity due to continued synthesis. Polyprenol monophosphate glucose and glucose lose ^{3}H-radioactivity in this period. Polyprenol monophosphate is probably saturated with [^{3}H]glucose after 30 min. Fast saturation of polyprenol monophosphate with glucose became evident from additional results from the kinetic experiments. The incorporation rate into polyprenol monophosphate declined already after 2 min at higher UDP-glucose concentrations. After addition of UDP-[^{14}C]glucose, [^{3}H]glucose in polyprenol monophosphate glucose might be substituted by an exchange reaction. We could not explain the disappearance of [^{3}H]glucose during the double-labelling period.

According to Parodi et al. (1972) the combination of characteristics, found for the polar lipid fraction, leads to the conclusion that polyprenol monophosphate glucose is the only labelled compound in this fraction. In our study, in which a discontinuous gradient of ammonium acetate is used, polyprenol monophosphate glucose appears to be eluted from the column by 99% methanol, containing 0.01 M ammonium acetate. In other investigations, in which linear gradients from 0 to 0.4 M ammonium acetate were used, polyprenol monophosphate glycosides were reported to be eluted at 0.125 M ammonium acetate (Garcia et al., 1974; Lezica et al., 1975,1976). The discrepancy between their results and ours is explicable by the fact that in our study the void volume of the column, which is about 85% of the column volume, may be left out of consideration. As no radioactive compounds were eluted from the column at 0.03 M to 0.4 M ammonium acetate, the presence of polyprenol pyrophosphate glycosides can be ruled out.

The combination of UDP-glucose:sterol glucosyl transferase and UDP-glucose:polyprenol monophosphate glucosyl transferase was reported in membrane systems of other plants such as *Pisum sativum* (Lezica et al., 1976) and *Phaseolus aureus* (Bowles et al., 1977). In these systems as in the membrane system, used in this study, sterol glucosides predominate, the remainder being polyprenol monophosphate glucose. In some cases the membranes, containing UDP-glucose:sterol glucosyl transferase activity were identified as Golgi membranes (Lercher and Wojciechowski, 1976; Bowles et al., 1977). Also the membrane fraction, used in this study, appeared to contain Golgi membranes (chapter 3).

The incorporation of about 23% of the radioactivity from the applied UDP-glucose into sucrose (Table 5.1) indicates that the membrane fraction contains sucrose synthetase activity. Sucrose synthetase is however thought to be a cytoplasmic

enzyme (Delmer, 1972, Baxter and Duffus, 1973). An interaction between β-glucan synthetase activity and sucrose synthetase activity was demonstrated in *Pisum sativum* by the incorporation of [^{14}C]glucose from [^{14}C]sucrose into β-glucans (Rollit and Maclachlan, 1974, Shore and Maclachlan, 1975) In young grape berries [^{14}C]sucrose appeared to be a good precursor for cellulose (Saito and Kasai, 1978) Sucrose synthetase activity in the membrane fraction from germinating *Petunia* pollen did not catalyse the synthesis of UDP-glucose or β-glucans with sucrose as a susbstrate. Sucrose therefore is probably synthesized by a combined activity of sucrose phosphate synthetase and sucrose phosphate phosphatase, which catalyse irreversible reactions (Pontis, 1978).

In most reports, concerning β-glucan synthesis *in vitro*, the radioactivity incorporated into newly-synthesized, alkali-insoluble material was taken as a measure for the enzymatic activity. The alkali-insoluble material contains, however, only β-glucans with a rather high degree of polymerization. For the kinetic and double-labelling experiments it is, therefore, necessary to obtain a fraction, that also contains cellodextrins of shorter chain length. Since the solubility of the shorter β-glucan chains is smaller in 80% methanol and chloroform-methanol than in alkali, this extraction procedure seemed to be more appropriate to obtain reliable values for the initial incorporation rates and the $^{3}H/^{14}C$ ratios in the β-glucans, although glycoproteins had to be removed

The classical way of obtaining evidence for the intermediate role of lipids in polysaccharide or glycoprotein synthesis is to incubate radioactive glycolipids, solubilised in Triton X-100, in the presence of the membranes and measure the amount of labelled polysaccharides or glycoproteins. Although various reaction conditions were investigated in this study, no enzymatic transfer of [^{14}C]glucose from polyprenol monophosphate glucose or the crude lipid preparation to β-glucans could be observed. The negative results of these experiments, however, do not rule out an intermediate role of these lipids in β-glucan synthesis. The

reaction conditions used might be unsuitable for such glycosyl transfer reactions.

In kinetic and double-labelling experiments the reaction conditions cannot be unsuitable for the synthesis of the lipid fractions, polyprenol monophosphate glucose and sterol glucosides, and the β-glucans since radioactivity is observed in these fractions. Because the β-glucans (pellet 2) show a higher initial incorporation rate in the kinetic experiments than sterol glucosides or polyprenol monophosphate glucose, these two (lipid) fractions cannot be intermediates in the synthesis of β-glucans in this *in vitro* system.

The results of the double-labelling experiments lead to the same conclusion. During the first 30 min of incubation with UDP-[^{3}H]glucose a pool of tritiated polyprenol monophosphate glucose, sterol glucosides and methanol-soluble and methanol-insoluble β-glucans is built up. Then UDP-[^{14}C]glucose is added, such that the $^{3}H/^{14}C$ ratio in UDP-glucose becomes about 2. Since the $^{3}H/^{14}C$ ratios in polyprenol monophosphate glucose and sterol glucosides approximate the value 2 more slowly than those in the β-glucans, with cellotriose as the only exception, polyprenol monophosphate glucose and sterol glucosides cannot be intermediates in the synthesis of β-glucans in this *in vitro* system.

Although the results of the kinetic and double-labelling experiments indicate that the *in vitro* synthesis of β-glucans takes place without intervention of one of the mentioned lipid fractions, the following possibilities may not be left out of consideration: 1. Another lipid, which fulfills the intermediate function in β-glucan synthesis, can have such a rapid turn-over and be present in such low quantities that its glucosylated derivative cannot be detected by the methods applied. 2. There might be two pathways in β-glucan synthesis, one without lipid-intermediate, evidently predominating in this *in vitro* system, and one with a lipid intermediate, which can be predominant in the *in vivo* situation.

Our results are not in agreement with those of Hopp et al.

(1978), who found a pathway for β-glucan synthesis in a particulate preparation from *Prototheca zopfii* via polyprenol monophosphate glucose, polyprenol pyrophosphate glucosides and glycoproteins. Their system is, however, different from the one reported here, e.g. the membrane fraction from *Petunia* pollen cannot use GDP-glucose for the synthesis of alkali-insoluble β-glucans (chapter 4).

The idea of involvement of a lipid intermediate was put forward, because in the *in vivo* situation the substrate for β-glucan synthesis (UDP-glucose or GDP-glucose) is supposed to be cytoplasmic. The active centre of the enzyme, involved in β-glucan synthesis, is supposed to be separated from this substrate by the plasma membrane or Golgi vesicle membrane, because β-glucans (cellulose) are extracellular polysaccharides. The lipid could serve as a carrier for glucose from substrate to active centre of the β-glucan synthetase. If there is no lipid intermediate involved, an alternative way would be given by a membrane-bound enzyme complex, containing UDP-glucose synthesizing enzymes. Berthillier and Got (1977a, 1977b) found membrane-bound UDP-glucose synthesizing enzymes in rat liver Golgi membranes.

The double-labelling experiments show, that cellotriose cannot be a precursor for cellotetraose and higher cellodextrins, because of its higher $^{3}H/^{14}C$ ratios at corresponding incubation times. So cellotriose does not fit in the pathway of chain lengthening in β-glucans. This is in agreement with the idea, postulated by Kjosbakken and Colvin (1973), that β-glucan chains might be elongated by addition of cellobiose units and not by addition of single glucose units, since in the β-glucan chains the glucose residues are rotated 180 degrees compared to their nearest neighbour. This poses a problem as to the stereospecificity of the enzyme that catalyses β-glucan synthesis. This problem may be solved by assuming chain lengthening in β-glucans by addition of cellobiose units. Neither polyprenol (pyro)phosphate [^{3}H]cellobiose nor UDP-[^{3}H]cellobiose could be observed after incubation of the

membrane fraction with UDP-[^{3}H]glucose. A protein intermediate as proposed for β-glucan synthetase activity in *Prototheca zopfii* (Hopp et al., 1978) can however not be excluded.

Electron microscopic autoradiography of β-glucans, synthesized by the membrane fraction

6.1. Introduction

Particulate β-glucan synthetase activity has been investigated in various ways. Most studies concern the biochemical characterization of enzymatic activity and reaction product (Péaud-Lenoël and Axélos, 1971; Van Der Woude et al., 1974). Others have examined the role of lipid intermediates (Kjosbakken and Colvin, 1973; Hopp et al., 1978). The presence and arrangement of possible β-glucan synthetase particles in the plasma membrane has also been extensively investigated (Burgess and Lindstead, 1976; Brown and Montezinos, 1976). In one investigation (Forge, 1977) the *in vitro* synthesis of cellulose microfibrils has been claimed.

This chapter deals with β-glucan synthetase activity studied by means of electron microscopic autoradiography of the reaction product. With this technique we intended to establish whether the newly-synthesized β-glucans were present as cellulose microfibrils.

6.2.1. Radioactivity and morphologic appearance of alkali-insoluble material after various treatments

Alkali-insoluble material, obtained by extraction without exogenous cellulose carrier, contains about 1.3% of the radioactivity originally added as UDP-[^{3}H]glucose to the membrane fraction (Table 6.1). Platinum-shadowed alkali-insoluble material (Fig. 6.1a) shows densily-packed, amorphous material in which microfibrils of about 10 nm are partly embedded.

Treatment with ultrasound does not remove any radioactivity (Table 6.1). However, the densily-packed amorphous material is disintegrated and more microfibrils are exposed (Fig. 6.1b). H_2O_2/CH_3COOH treatment of control or sonicated

Table 6.1. Influence of various treatments of alkali-insoluble material on the radioactivity (dpm) and on the morphologic appearance of the remaining insoluble pellets. Membrane material, equivalent to 10 mg protein, was incubated for 90 min with 100 µCi UDP-[^{3}H]glucose (2.2.1).

Treatment	Dpm (x 10^{-6}) in insoluble pellet	Morphologic appearance
None	2.8	Microfibrils, partly embedded in amorphous material (Fig. 6.1a)
Ultrasound	2.8	Many loose microfibrils between dispersed amorphous material (Fig. 6.1b)
H_2O_2/CH_3COOH	1.1	Less amorphous material, more microfibrils (Fig. 6.1c)
Ultrasound + H_2O_2/CH_3COOH	1.4	Less amorphous material, more microfibrils (Fig. 6.1d)

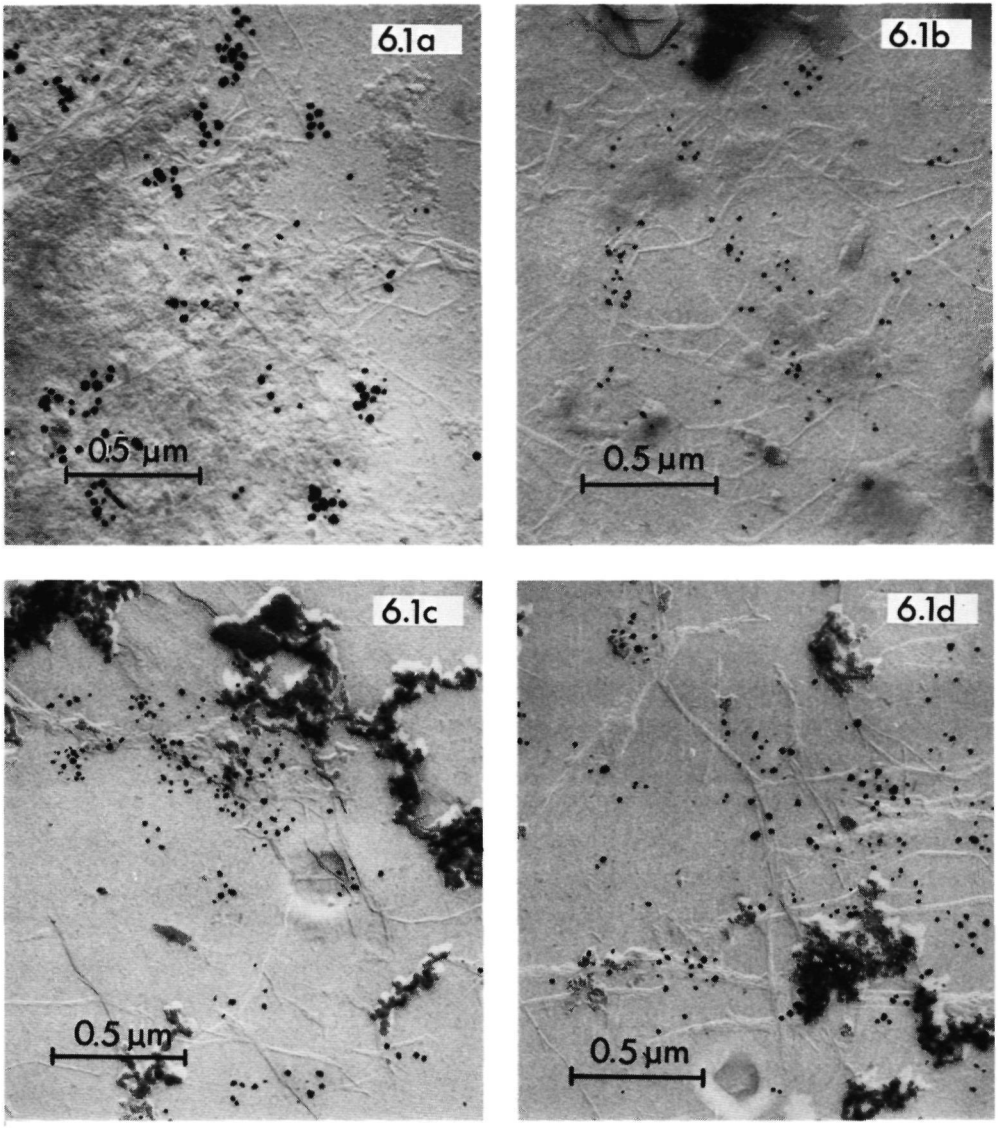


Fig. 6.1. EM autoradiographs of alkali-insoluble material a. untreated and after treatment with b. ultrasound, c. H_2O_2/CH_3COOH and d. ultrasound, followed by H_2O_2/CH_3COOH. Exposure time: 14 days. Magnification 36,000 x

Table 6.2. Evaluation of 30 EM-autoradiographs of alkali-insoluble material a. untreated and after treatment with b. ultrasound, c. H_2O_2/CH_3COOH and d. ultrasound + H_2O_2/CH_3COOH.
- = not detected. Empty = empty space, amorph = amorphous material, fibrils = microfibrils

(Junctional) features	Number of silver grain groups				Number of circles				Silver grain groups/area			
	a	b	c	d	a	b	c	d	a	b	c	d
Empty	38	15	22	73	2671	2239	2066	2616	0.014	0.007	0.011	0.028
Amorph	134	15	69	72	157	139	322	67	0.85	0.11	0.21	1.07
Empty-amorph	38	29	189	60	165	373	510	106	0.23	0.08	0.37	0.57
Empty-fibrils	5	27	46	227	6	39	44	153	0.83	0.69	1.05	1.48
Amorph-fibrils	9	-	-	13	-	-	-	4	∞	-	-	3.25
Empty-amorph-fibrils	1	24	103	127	15	10	57	52	15.0	2.4	1.81	2.44

alkali-insoluble material removes about 55% of the radioactivity from the insoluble pellets (Table 6.1). Electron micrographs of these pellets show less amorphous material and more microfibrils in comparison to the untreated alkali-insoluble material (Figs. 6.1).

6.2.2. Electron microscopic autoradiography of alkali-insoluble material after various treatments

The autoradiographs of the insoluble pellets, remaining after the various treatments of alkali-insoluble material, show an accumulation of silver grain groups above microfibrils and amorphous material (Figs. 6.1). Also without incubation or after incubation of denatured membranes with UDP-[^{3}H]glucose, microfibrils, partly embedded in amorphous material, are observed. However, there is no accumulation of silver grain groups above any feature. The alkali-insoluble material, thus obtained, does not contain radioactivity.

6.2.3. Statistical evaluation of the autoradiographs

Statistical evaluation of the autoradiographs (Table 6.2) confirms that the accumulation of silver grain groups above any feature which contains amorphous material and/or microfibrils is higher than that above the feature empty space ($p<10^{-6}$). The silver grain groups/area ratios above the junctional items empty space-microfibrils are significantly higher than those above empty space-amorphous material ($p = 0.085-<10^{-6}$), indicating a higher accumulation of silver grain groups above microfibrils compared to above the amorphous material. The differences between the junctional items empty space-microfibrils and empty space-amorphous material-microfibrils are also smaller and less significant ($p = 0.009-0.055$) than the differences between empty space-amorphous material and empty space-amorphous material-microfibrils ($p<10^{-6}$). An additional result appearing from comparison of

micrographs is that the silver grain groups above amorphous material are heterogenously distributed. Silver grain groups accumulate more above amorphous material from which microfibrils project. There is no significant influence of the treatments with ultrasound and/or H_2O_2/CH_3COOH on the silver grain groups/area ratios.

6.3. Discussion

Electron microscopy of platinum-shadowed alkali-insoluble material shows amorphous material and microfibrils. Treatment with ultrasound separates these two structural features, exposing more microfibrils. H_2O_2/CH_3COOH treatment also exposes more microfibrils by solubilizing amorphous material. Like Engels (1974b) we did not observe microfibrils in an unextracted membrane fraction from *Petunia* pollen tubes. Engels stated that this might be due to the fact that cellulose would be masked by protein and/or lipid which prevents its crystallization. In our opinion the microfibrils are present in the membrane fraction as contamination but dilution with membranous material minimalises the chance of their observation. After removal of membranous material with chloroform and alkali Engels (1974b) could obtain an X-ray diffraction pattern indicating the presence of cellulose. This is in good agreement with our observation of microfibrils after a similar extraction.

Amorphous material and microfibrils appear to act as carriers for newly-synthesized radioactive material during the extraction procedure. From the radioactivity in UDP-[^{3}H]glucose about 1.3% is found again in the alkali-insoluble material. With the addition of cellulose after incubation 4% incorporation was measured in a similar experiment (chapter 4). This exogenous cellulose may bind β-glucan chains and thus increase the radioactivity in alkali-insoluble material.

The nature of the alkali-insoluble, amorphous material is unknown. Hence it remains obscure how it interacts with the newly-synthesized radioactive material which causes the accumulation of

silver grain groups above it. One part might be simply trapped by the amorphous material. The radioactive material may be solubilized together with the amorphous material by the H_2O_2/CH_3COOH treatment. This could explain the removal of radioactivity from the insoluble pellet by this treatment. Another part of the radioactive material, causing silver grain groups above the amorphous material, might be linked to embedded and hence invisible microfibrils. This suggestion is supported by the more prominent accumulation of silver grain groups above amorphous material from which microfibrils project.

From the evaluation of the autoradiograhs it appears that the microfibrils show the highest accumulation of silver grain groups. This indicates a high affinity of the radioactive material for these structures. In chapter 4 it was shown that the radioactive, alkali-insoluble material consisted of β-1,3- and β-1,4-glucans. It is very likely that such β-glucans interact with cellulose microfibrils by means of hydrogen bonds. Such bonds also cause the crystallization of β-1,4-glucan chains to cellulose microfibrils (Frey-Wyssling, 1976, pp. 93-94).

Whether these radioactive β-glucans interact with the microfibrils during the incubation, the extraction procedure or during drying of the sample droplets cannot be concluded from these experiments. An interaction during the incubation could convert short and hence alkali-soluble chains of β-glucans into insoluble ones. Since alkali-insoluble material is not labelled upon incubation of denatured membranes with UDP-[^{3}H]glucose a certain chain length of newly-synthesized β-glucans should be a prerequisite for such an interaction.

No accumulation of silver grain groups is observed specifically above the microfibrillar ends. Thus, a primer function of the microfibrils appears improbable. Cellulose present endogenously or added exogenously, therefore, more likely functions as carrier for newly-synthesized β-glucans.

If a microfibril would be entirely newly-synthesized from UDP-[^{3}H]glucose with a specific activity of 3.7 Ci/mmole, the silver grain groups/area ratio above such a structure were to be

calculated as follows 3.7 Ci/mmole means 1 disintegration per 2.7×10^3 glucose residues in two weeks. An efficiency of 33% of the applied autoradiographic procedure (Vrensen, 1970b) would cause 1 silver grain group per 8.1×10^3 glucose residues which is equivalent to 1 silver grain group per 4 µm single β-glucan chain. According to Frey-Wyssling (1976, p 18) a cellulose microfibril consists of about 1,000 parallel β-glucan chains. Therefore, 1,000 silver grain groups should be present per 4 µm microfibril. The autoradiographs show that this is certainly not the case. It would be valuable if claims of *in vitro* synthesis of microfibrils (Forge, 1977) were checked in this way

The following reasons might explain why the *in vitro*-synthesized β-glucans do not crystallize to cellulose microfibrils.

1. The newly-synthesized β-glucans might be precursors for other polysaccharides such as xyloglucans, a constituent of the amorphous matrix of the plant cell wall (Albersheim, 1978). These xyloglucans also interact with the cellulose microfibrils in order to connect them with the cell wall matrix (Albersheim, 1973).
2. The newly-synthesized β-glucans may require a special mechanism for crystallization, e.g. a linear arrangement of the synthesizing particles (Brown and Montezinos, 1976, Forge, 1977). This arrangement can be disturbed by the homogenization of the pollen tube.
3. There are much more contaminating microfibrils than *in vitro* synthesized β-glucans Therefore, these *in vitro* synthesized β-glucans crystallize with the microfibrils already present before they can crystallize with each other.

Chapter 7

General discussion

7.1. Role of Golgi vesicles in cell wall synthesis

The HIO_4-PTA/CrO_3 staining technique applied on *Petunia* pollen tubes appeared to be a convenient method to study the role of Golgi vesicles in plant cell wall synthesis. Because of the abundancy of these vesicles, the 'maturing' from non-stained dictyosomal membranes to Golgi vesicle membranes, with contrast similar to that of the plasma membrane, could be easily followed. The increase in contrast of the Golgi vesicle content may be related to a possible synthesis of polysaccharides inside the Golgi vesicles (Pease, 1970). The fusion of the Golgi vesicle membrane with the plasma membrane is facilitated by the biochemical similarity of both membrane types which is a consequence of the maturing process. By the fusion the plasma membrane is extended and the Golgi vesicle content, probably cell wall precursors, is extruded and added to the cell wall. It seems worthwile to investigate in this way also other cell types showing tip growth such as root hairs, fungal hyphae and fern rhizoids. Earlier observations of a positive reaction of Golgi vesicles on the HIO_4-PTA/CrO_3 staining were reported by Roland (1973) and Montezinos and Brown (1976), but in their systems Golgi vesicles were less abundant. Concerning the nature of the reacting polysaccharides no definitive conclusions can be drawn.

7.2. Characterization of the membrane fraction

After staining with lead citrate (Fig. 3.2a) or $KMnO_4$ (Figs. 4.1a and 4.1b) the membrane fraction used in our studies appeared to be very similar to the membrane fraction, identified by Engels

(1973) as pure Golgi vesicles. The presence of Golgi vesicles in the membrane fraction is indicated by the presence of small vesicles with electron-dense membrane and content after HIO_4-PTA/CrO_3 staining, latent IDPase (chapter 3) and UDP-glucose: steryl glucosyl transferase activities (chapter 5). Enzyme activities characteristic for other than Golgi membranes and the presence of vesicles without a HIO_4-PTA/CrO_3-positive content indicate that this fraction does not contain exclusively Golgi vesicles.

Nevertheless, the membrane fraction was still the most appropriate fraction to study β-glucan synthetase activity, because the determination of marker enzyme activities showed a relatively small amount of endoplasmic reticulum and mitochondrial membranes. The presence of vesicles with HIO_4-PTA/CrO_3-positive membranes and -negative content (plasma membranes) or -positive membrane and content (Golgi vesicles) indicates that β-glucan synthetase activity in the membrane fraction can be due to Golgi membranes and to plasma membranes. A comparison of distribution patterns of K^+ stimulated Mg^{2+} ATPase, latent IDPase (chapter 3) and β-glucan synthetase activities (chapter 4) also reveals that β-glucan synthetase activity in the membrane fraction may be due to plasma membranes as well.

7.3. Characterization of β-glucan synthetase activity

The characterization of β-glucan synthetase activity in the membrane fraction was necessary to establish the optimal conditions for further experiments. An optimum pH of about 8, an optimum temperature of 25-30°C and a higher activity in TRIS than in phosphate buffer are not uncommon for particulate β-glucan synthetase activities (Pinsky and Ordin, 1969; Larsen and Brummond, 1974).

The specificity for UDP-glucose of the β-glucan synthetase activity is less common. Most β-glucan synthetase systems of higher plants can also use GDP-glucose as substrate (Chambers and Elbein, 1970; Robinson and Preston, 1972, Hopp et al., 1978).

β-Glucan synthetase activities from *Lilium longiflorum* pollen tubes (Southworth and Dickinson, 1975) and from yeast (López-Romero and Ruiz-Herrera, 1977) are also specific for UDP-glucose, but in these systems only β-1,3-glucans are synthesized.

7.4. Other enzymatic activities which use UDP-glucose as substrate

In addition to the β-glucan synthetase, which catalyses the incorporation of glucose, other enzymes in the membrane fraction use UDP-glucose as substrate and catalyse the incorporation of glucose into polyprenol monophosphate glucose, sterol glucosides and sucrose (chapter 5). Solubilization of radioactivity from pellet 1 with pronase suggests some glucose incorporation into glycoproteins. The pronase preparation, however, might be contaminated with glycosidases, so it remains questionable whether radioactive glycoproteins are digested.

Polyprenol monophosphate glucose and steryl glucosides appear to have no obligatory intermediate function in β-glucan synthesis in this system. The mechanism of transfer of activated glucose through the membrane to the active site of β-glucan synthetase remains obscure. A reversed sucrose synthetase reaction: sucrose + UDP → fructose + UDP-glucose, provides a possible transfer mechanism. Although such a reaction was not found in this *in vitro* system it might function *in vivo*. Attempts to stimulate β-glucan synthetase activity through inhibition of sucrose synthesis failed.

The fact that cellotriose is not a precursor for cellotetraose (chapter 5) suggests the possible occurrence of a cellobiose intermediate in β-glucan synthetase activity. Neither polyprenol monophosphate cellobiose nor polyprenol pyrophosphate cellobiose nor UDP-cellobiose were detected in the incubation medium. The involvement of a protein intermdiate in β-glucan synthesis, as suggested by Hopp et al. (1978), was not investigated but may be another possibility.

Alkali-insoluble material, obtained by extraction of the non-incubated membrane fraction, contains besides amorphous material also microfibrils (chapter 6). The observation of microfibrils might be explained by crystallization of originally masked cellulose molecules as proposed by Engels (1974b). To my opinion a contamination of the membrane fraction with cell wall material seems more probable.

Upon incubation of the membrane fraction with UDP-[^{3}H]glucose labelled β-glucans are synthesized which show a strong interaction with the microfibrils (chapter 6). The formation of β-1,3- together with β-1,4-glucosidic linkages (chapter 4) is quite common in studies of β-glucan synthesis *in vitro*, especially when higher UDP-glucose concentrations are applied during incubation (Péaud-Lenoël and Axélos, 1971; Van Der Woude et al., 1974; Raymond et al., 1978). In our system this observation indicates the synthesis of two different types of β-glucans, one similar to cellulose with only β-1,4-glucosidic linkages and one similar to callose with only β-1,3-glucosidic linkages. Both β-glucans were detected in *Petunia* pollen tube walls (Engels, 1974b; Cresti et al., 1976). A single β-glucan with β-1,3- as well as β-1,4-glucosidic linkages appears improbable because a trisaccharide with both types of linkages was not observed in the hydrolysate of alkali-insoluble material.

Because of their interaction with the microfibrils the morphology of the individual, newly-synthesized β-glucan chains remains obscure. Explanations for the fact that the newly-synthesized β-glucans do not crystallize with each other to microfibrils might be: 1) the disturbance of the mechanism for crystallization, e.g. linearly arranged particles in the membrane (Brown and Montezinos, 1976; Mueller et al., 1976), by homogenization of the pollen tube, 2) the β-glucans "crystallize with the microfibrils already present before they can crystallize with each other, 3) the newly-synthesized β-glucans are precursors for xyloglucans and the synthesis of β-glucans for cellulose microfibrils does not take place

in this membrane fraction.

The small effect of the H_2O_2/CH_3COOH treatment indicates a strong interaction of the newly-synthesized β-glucans with the microfibrils. There might be a parallel between such an interaction and that which causes the synthesis of cellulose microfibrils by crystallization of nascent stage cellulose molecules as proposed by Leppard et al. (1975, 1978), Mueller et al. (1976) and Colvin and Leppard (1977).

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Cellulose occurs as a fibrillar polysaccharide in the cell walls of most plants. It is composed of parallel arranged, linear polymers of β-1,4-glucosidic linked glucose residues. This thesis deals with an investigation of the synthesis of these β-1,4-glucans by a membrane fraction isolated from germinating *Petunia* pollen.

The membrane fraction was identified on morphological basis to contain Golgi vesicles (chapter 4). Application of the Roland stain with HIO_4-PTA/CrO_3 and assay of marker enzyme activities indicate that also membranes originating from other cell organelles are present in this fraction (chapter 3). The Roland stain was a convenient method to demonstrate a role of Golgi vesicles in the synthesis of cell walls. Evidence was obtained for a maturing process of the Golgi vesicle membrane and the synthesis of cell wall precursors within the Golgi vesicles.

The membrane fraction contained β-glucan synthetase activity that catalysed the incorporation of glucose from UDP-glucose and not from GDP-glucose into alkali-insoluble β-glucans (chapter 4). Other enzymes in the membrane fraction catalysed the incorporation of glucose from UDP-glucose into polyprenol monophosphate glucose, sterol glucosides, sucrose and possibly glycoproteins (chapter 5). Neither polyprenol monophosphate glucose nor sterol glucosides are obligatory intermediates for β-glucan synthesis. NADPH inhibited sucrose synthesis, but did not influence β-glucan synthesis.

The newly-synthesized β-glucans contain β-1,3- as well as β-1,4-glucosidic linkages (chapter 4). They have a great affinity for microfibrils that contaminate the membrane fraction (chapter 6).

Cellulose is een polysaccharide, dat voorkomt in de vorm van fibrillen in de celwanden van de meeste planten. Het is opgebouwd uit parallel gerangschikte, lineaire polymeren van β-1,4-glucosidisch gebonden glucose-residuen. Dit proefschrift beschrijft een onderzoek naar de synthese van deze β-1,4-glucanen door een membraanfractie, geïsoleerd uit *Petunia* pollenbuizen.

De in deze fractie waargenomen membranen werden op morphologische gronden gekarakteriseerd als Golgiblaasjes (hoofdstuk 4). Toepassing van de Roland-kleuring met HIO_4-PTA/CrO_3 en bepaling van markerenzym activiteiten toonden echter aan, dat ook andere membraantypen in deze fractie aanwezig waren (hoofdstuk 3).

De Roland-kleuring met HIO_4-PTA/CrO_3 bleek een goede methode om een rol van Golgiblaasjes bij de synthese van celwandmateriaal in pollenbuizen aan te tonen. Aanwijzingen werden verkregen voor een rijpingsproces van de membraan van het Golgiblaasje en voor een synthese van celwandprecursors binnen de blaasjes.

In deze membraanfractie werd β-glucaansynthetase activiteit aangetoond, die de overdracht van glucose uit UDP-glucose, maar niet uit GDP-glucose, katalyseerde (hoofdstuk 4). Andere enzymen in de membraanfractie katalyseerden de overdracht van glucose uit UDP-glucose naar polyprenolmonophosphaatglucose, sterolglucosiden, sucrose en mogelijk glycoproteïnen (hoofdstuk 5). Polyprenol monophosphaatglucose noch sterolglucosiden bleken obligate intermediairen in de β-glucaansynthese. NADPH remde de sucrosesynthese, maar had geen invloed op de β-glucaansynthese.

De nieuw-gesynthetiseerde β-glucanen bevatten zowel β-1,3- als β-1,4-glucosidische bindingen (hoofdstuk 4). Zij vertoonden een hoge affiniteit voor de microfibrillen, die als contaminatie in de membraanfractie aanwezig waren (hoofdstuk 6).

Helsper J.P.F.G., Groot N.J. (1976): Reaction of phosphotungstic acid-chromic acid on plasma membranes and Golgi vesicles in pollen tube tips of *Petunia hybrida*. Ultramicroscopy 2, 127-128

Helsper J.P.F.G., Veerkamp J.H., Sassen M.M.A. (1977): β-Glucan synthetase activity in Golgi vesicles of *Petunia hybrida*. Planta 133, 303-308

Helsper J.P.F.G. (1979): The possible role of lipid intermediates in the synthesis of β-glucans by a membrane fraction from pollen tubes of *Petunia hybrida*. Planta 144, 443-450

Helsper J.P.F.G., Van Kuppevelt A.H.M.S.M., Groot N.J. (1979): Cytochemical staining and marker enzyme activities in membranes from pollen tubes of *Petunia hybrida*. Submitted to Planta

Helsper J.P.F.G. (1979): Electron microscopic autoradiography of β-glucans, synthesized by a membrane fraction from pollen tubes of *Petunia hybrida*. Submitted to Planta

Curriculum vitae

Johannes P.F.G. Helsper werd geboren te Helmond op 13 juni 1950. Hij behaalde het eindexamen H.B.S.-B in 1967 (Dr. Knippenberg-college te Helmond) en begon in datzelfde jaar zijn studie Biologie aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen (B_4) werd behaald in mei 1971 en het doctoraal-examen in december 1974 met als hoofdrichting Biochemie (Prof. Dr. H. Bloemendal) en als bijvakken Botanie (Prof. Dr. H.F. Linskens) en Submicroscopische Morphologie van de Plant (Dr. M.M.A. Sassen). In februari 1975 startte hij een onderzoek aan het Laboratorium voor Submicroscopische Morphologie van de Plant te Nijmegen over de β-glucaansynthetase activiteit in membraansystemen, geïsoleerd uit *Petunia* pollenbuizen. Vanaf september 1975 werd dit een promotie-onderzoek onder leiding van Dr. M.M.A. Sassen en Dr. J.H. Veerkamp

Stellingen

behorende bij het proefschrift

β-GLUCAN SYNTHESIS
BY A MEMBRANE FRACTION
FROM GERMINATING PETUNIA POLLEN

21 juni 1979

I

De HIO_4-PTA/CrO_3 kleuring volgens Roland et al is een geschikte methode om de rol van Golgi-blaasjes in de celwandsynthese te bestuderen

Roland J -C., Lembi C.A., Morré D J. (1972) Stain Technol. 47 195-200

Dit proefschrift, hoofdstuk 3

II

De blaasjesfractie, geïsoleerd volgens Engels uit kiemende pollenbuizen als de 0.7-0 9 M sucrosefractie, bestaat niet alleen uit Golgiblaasjes

Engels F.M. (1973)· Acta Bot. Neerl. 22, 6-13

Dit proefschrift, hoofdstuk 3 en hoofdstuk 4

III

De bewering, dat polyprenol monophosphaat glycosiden van DEAE-cellulose acetaat geeluëerd worden met 99% methanol dat tenminste 0,125 M ammoniumacetaat bevat, is onjuist

Garcia R.C., Recondo E., Dankert M. (1974)· Eur. J. Biochem. 43, 93-105

Dit proefschrift, hoofdstuk 5

IV

Bij de synthese van β-glucanen is niet noodzakelijkerwijze een lipide-intermediair betrokken

Dit proefschrift, hoofdstuk 5

V

De toename in β-glucaan synthetase activiteit, zoals die werd waargenomen door Ray na toediening van sucrose aan erwtenweefsel, kan het gevolg zijn van repressie van sucrosesynthese
Ray P.M. (1973). Plant Physiol. 51, 601-608

VI

De 'multinet growth hypothesis' van Roelofsen en Houwink en de 'ordered fibril hypothesis' van Roland et al. spreken elkaar niet noodzakelijkerwijze tegen
Roelofsen P.A., Houwink A.L. (1953). Acta Bot. Neerl. 2, 218-225
Roland J.-C , Vian B., Reis D (1975) J. Cell Sci. 19, 239-259

VII

De Km-waarde voor UDP-galactose diacylglycerol-galactose transferase, zoals die door Blee en Schantz wordt berekend, berust op resultaten die een dergelijke berekening niet rechtvaardigen
Blee E., Schantz R. (1978): Plant Sci. Lett. 13, 247-255

VIII

De term incompatibiliteit, indien gebruikt voor mechanismen die een succesvolle bevruchting bij planten verhinderen, is dubbelzinnig. Men dient onderscheid te maken in S-gen afhankelijke incompatibiliteit en incongruentie

IX

De conclusies omtrent de verschillen in samenstelling tussen basaalmembranen van niertubuli en glomeruli, zoals die worden getrokken worden door Mahieu en Winand, worden niet gedekt door de vermelde resultaten
Mahieu P., Winand R.J. (1970)· Eur. J. Biochem. 12, 410-418

X

Bij het leggen van een correlatie tussen de afgelegde afstand tijdens een 12 minutenlooptest volgens Cooper en de maximale zuurstofopname per minuut, dient men rekening te houden met de weersomstandigheden en met de tak van sport die door de proefpersoon regelmatig beoefend wordt.
Cooper K.H. (1968): J.A.M.A. 203, 201-204

XI

Door de reklame voor geneesmiddelen aan banden te leggen kan op de kosten voor de gezondheidszorg bezuinigd worden

XII

De symboolinformatie op Nederlandse spoorwegstations zou aanmerkelijk verbeterd kunnen worden door erbij te vermelden wat de symbolen betekenen